The Role of SOX2 in Pituitary Development

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I, Sam G J Goldsmith, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

SOX2 is expressed in the pituitary anlagen, Rathke's Pouch (RP), from its induction at 9.0dpc. RP progenitors initially expand, later giving rise to all pituitary cell types. As cells differentiate, SOX2 expression is down-regulated, but a population of SOX2^{+ve} cells persists until adulthood. These represent adult stem cells, displaying regenerative potential upon physiological demand. In humans and mice, heterozygous loss-of-function mutations in *SOX2*/*Sox2* are associated with hypopituitarism. It was therefore decided to investigate the role of SOX2 during murine pituitary development.

Homozygous null mutations of *Sox2* lead to embryo lethality following implantation; consequently conditional strategies were used to delete the gene specifically in RP. *Nkx3.1^{Cre}* and *FoxG1^{Cre}*, display different spatio-temporal patterns of activity in RP. The severity in hypoplasia and reduced progenitor cell proliferation correlated with the efficiency and timing of Cre-mediated deletion of *Sox2*. The expression of the transcription factor SIX6, known for its role in promoting cell proliferation, was downregulated. Conversely expression of the cell cycle inhibitor p27kip1 is up-regulated, in the absence of *Sox2*. Furthermore, the proliferation defect in *Sox2* mutants can be rescued by homozygous loss of *p27kip1*, demonstrating a genetic interaction. This suggests that SOX2 promotes progenitor cell proliferation in the early RP and may do so, at least in part, by regulating *Six6* and *p27kip1* expression.

Sox2 RP mutants display a disproportionate reduction in melanotroph cell numbers in the intermediate lobe (IL). The *Sox2*-deleted cells display a downregulation of the melanotroph lineage specifier, PAX7. Consequently, the few differentiated cells present in mutant IL switch from a melanotroph identity to a corticotroph fate, despite these cells never being present in the normal IL. This phenotype was not rescued in *p27kip1;Sox2* compound mutants. This suggests that SOX2 plays two independent roles during

pituitary development, initially promoting progenitor proliferation and later specifying IL melanotroph cell fate.

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Abbreviations

ACTH	Adrenocorticotropic Hormone
Adx	Adrenalectomies
AL	Anterior Lobe
AP	Anterior Pituitary
BB	Blocking Buffer
bHLH	basic Helix-Loop-Helix
BMP	Bone Morphogenetic Protein
bp	base pairs
BrdU	Bromodeoxyuridine
CDKI	Cyclin Dependent Kinase Inhibitor
CNS	Central Nervous System
CPHD	Combined Pituitary Hormone Deficiency
CRH	Corticotropin Releasing Hormone
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dpc	days post coitum
E	Embryonic day
EdU	Ethynyl-2-deoxyuridine
EGR-1	Early Growth Response 1
ES	Embryonic Stem
eYFP	Enhanced Yellow Fluorescent Protein
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
ECR	Evolutionary Conserved Region
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptor
FGF	Fibroblast Growth Factor
FOXG1	Forkhead Box G1
FS	Folliculostellate
FSH	Follicle-Stimulating Hormone

GAS1	Growth Arrest-Specific 1
GATA2	GATA Binding Protein 2
GBS	Gli Binging Site
Gdx	Gonadectomies
GFRα2	Glial cell line-derived neurotropic factor (GDNF) receptor alpha 2
GFP	Green Fluorescent Protein
GH	Growth Hormone
GHRH	Growth-Hormone Releasing Hormone
GnRH	Gonadotropin-Releasing Hormone
GnRHr	Gonadotropin-Releasing Hormone receptor
GPS	GFRα2/PROP/Stem
HCL	Hydrochloric Acid
HESX1	Homeobox Expressed in ES Cells 1
HMG	High Mobility Group
HPE	Holoprosencephaly
ICM	Inner Cell Mass
IL	Intermediate Lobe
iPS	induced Pluripotent Stem
ISL1	Insulin gene enhancer protein 1
Kb	Kilobase
LCC	Light Coat and Circling
LH	Lutenising Hormone
LHX3	LIM Homeobox 3
LHX4	LIM Homeobox 4
M	Molar
MC	Marginal Cell
mM	Millimolar
NKX2.1	NK2 Homeobox 1
NKX3.1	NK3 Homeobox 1
MSH	Melanocyte-Stimulating Hormone
NaCl	Sodium Chloride
NeuroD1	Neuronal Differentiation 1

NR51a	Nuclear Receptor 5a1
NSC	Neural Stem Cell
PAX6	Paired Box 6
PAX7	Paired Box 7
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline Triton
PCR	Polymerase Chain Reaction
PD	Paired Domain
PFA	Paraformaldehyde
PIT1	Pituitary Specific Transcription Factor 1
PITX1	Paired-Like Homeodomain 1
PITX2	Paired-Like Homeodomain 2
PL	Posterior Lobe
POMC	Pro-opiomelanocortin
POU1f1	POU Class 1 Homeobox 1
PP	Posterior Pituitary
PrL	Prolactin
PROP1	Paired-like Homeobox 1
PTC1	Patched 1
qPCR	quantitate Polymerase Chain Reaction
R26R	Rosa26 Reporter
RP	Rathke's Pouch
SC	Stem Cell
SCA1	Stem Cell Antigen 1
SD	SIX Domain
SF1	Steroidogenic Factor 1
SHH	Sonic Hedgehog
SIX3	SIX Homeobox 3
SIX6	SIX Homeobox 6
SK1	Schedule 1 Kill
SOD	Septo-Optic Dysplasia
SOX	SRY (Sex determining region T)-box
SOX2	SRY (Sex determining region T)-box 2

SOX9	SRY (Sex determining region T)-box 9
SP	Side Population
SRY	Sex-determining region Y
TAE	Tris-acetate-EDTA
TBX19	T-Box 19
TF	Transcription Factor
TFBS	Transcription Factor Binding Sites
TLE1	Transducin-Like Enhancer of Split 1
TPIT	T-Box Factor Pituitary
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
UTF1	Undifferentiated Transcription Factor 1
VD	Ventral Diencephalon
WT	Wild Type
WNT	Wingless-Type
YSB	Yellow Submarine
α-GSU	Glycoprotein Hormones, α-Subunit
β	Beta
°c	Degrees Centigrade
µl	Micro Liters
µm	Micrometre
%	Percent

1. General Introduction

The studies presented in this thesis aim to examine the role SOX2 plays in embryonic pituitary progenitors. Throughout this chapter I will introduce the topics studied in the thesis. Moreover I will outline the aims of the thesis in addition to discussing previously published data that helped form some of the hypothesis we used as the basis of this study.

1.1 SOX2: a stem cell transcription factor

The founding member of the *Sox* gene family, encoding for transcription factors (TFs), and with which all 19 other mammalian *Sox* genes share at least 50% homology, is *Sry* (Sex-determining region Y). Discovered in 1990, SRY is solely responsible for the primary male characteristics associated with the Y chromosome (Gubbay et al., 1990; Koopman et al., 1991; Sinclair et al., 1990). Containing only one exon, *Sry* codes for a protein containing a high mobility group (HMG) domain, which is well conserved, while the rest of the protein displays high variability between mouse and human (Gubbay et al., 1990) and is poorly conserved amongst other animals. This HMG domain specifically binds to the minor groove of DNA, resulting in bending of the DNA through 90-120°. This bending of the DNA is critical for the correct function of SRY. Mutation of the protein to prevent this bending results in male to female sex reversal (Gubbay et al., 1990; Koopman et al., 1991; Pontiggia et al., 1994).

The SOX (Sex determining region Y)-box of TFs comprises nine distinct groups (A-H), based on sequence homology. SRY is the sole member of group A. Other *Sox* genes are mostly highly evolutionarily conserved, with two additional groups being identified in *Xenopous* (*SoxI*) and *C.elegans* (*SoxJ*) (Bowles er al., 2000). Moreover the evolutionary origin of *Sox* genes may go back even further with the more recent discovery of *Sox*-like genes in single cell organisms (*Monosiga brevicollis*) (King et al., 2008). Nevertheless, the defining characteristic shared between all *Sox* genes is the

high sequence similarity within the HMG domain. Consequently all *Sox* genes recognise the transcription factor binding motif [(A/T)(A/T)CAA(A/T)G] (Harley et al., 1994; van de Wetering et al., 1992; Wright et al., 1993).

Sox2 (SRY-like HMG box containing gene 2), was discovered along with the other *SoxB1* genes (*Sox1* & *Sox3*) at the same time as *Sry* (Figure 1.1) (Gubbay et al., 1990). Located on chromosome 3 in human and mouse genome, *Sox2* like *Sry* consists of only one exon coding for a TF including the HMG box and a C-terminal transactivation domain (Figure 1.1) (Gubbay et al., 1990; for review see Kamachi & Kondoh., 2013).

1.1.1 SOX2 and Embryonic Stem (ES) cell pluripotency

Sox2 mRNA transcription starts in the mouse embryo morula at 2.5 days post coitum (dpc), but in fact the protein and its messenger are present from fertilization, as they are stored in the oocyte (Avilion et al., 2003; Keramari et al., 2010). SOX2 is essential for development to progress, as *Sox2* deletion is lethal at peri-implantation stages. This is due to failure to maintain development of the epiblast and extra-embryonic ectoderm, identified through the reduction in OCT4 expression and absence of *Ffg4*, a target gene of SOX2 (Avilion et al., 2003; Yuan et a., 1995). The presence of maternal SOX2 was suggested to delay the appearance of the embryonic *Sox2* deletion phenotype in the inner cell mass (ICM) (Avilion et al., 2003). More recently it was observed that knock down of both maternal and embryonic *Sox2* mRNAs by siRNA at the two-cell stage results in an even earlier arrest in development, at the morula stage (Keramari et al., 2010).

The ICM in the blastocyst contains the pluripotent cells from which the extraembryonic endoderm and all embryonic tissues are derived. ES cells are derived from isolated ICMs (Evans & Kaufman., 1981; Martin., 1981). They retain their pluripotent state thanks to the maintenance of expression of the 'core' pluripotency factors, SOX2, OCT4 and NANOG (Chambers et al., 2003; Masui et al., 2007; Niwa et al., 2000). SOX2 is also one of the four 'Yamanaka factors' used to reprogram terminally differentiated cells into

induced pluripotent stem (iPS) cells, along with OCT3/4, c-MYC and KLF4 (Takahashi & Yamanaka., 2006; Takahashi et al., 2007). These core pluripotency factors restore an ES like state through the upregulation of genes associated with totipotency such as members of Wnt and TGFβ pathways (James etal., 2005; Sato et al., 2004) and chromatin modification (Bagci & Fisher., 2013). Moreover they also promote their own expression in a positive feedback loop (Catena et al., 2004; Okumura-Nakanishi et al., 2005; Rodda et al., 2005). Conversely, SOX2, NANOG and OCT4 promote the downregulation of markers of differentiation, including HOXB1, PAX6 and MYF5 (Boyer et al., 2010). It was recently shown that iPS cells can be derived from mice deleted for the cell cycle regulator cyclin dependent kinase inhibitor (CDKI) *p27kip1* without exogenous addition of *Sox2* (Li et al., 2012). Moreover, *p27kip1* null iPS cells are unable to differentiate, and this is associated with persistence of SOX2 protein (Li et al., 2012). Li et al (2012) suggested p27kip1 antagonises *Sox2*, and it was demonstrated that p27kip1 directly binds to a *Sox2* enhancer, allowing for the recruitment of the p130-E2F4-SIN3A repression complex (Li et al., 2012). Consequently, upregulation of p27kip1 during ES/iPS cell differentiation induces SOX2 down-regulation and this is required for differentiation to occur.

Figure 1.1: SOXB subgroup of transcription factors. The SOXB1 group of TFs consists of SOX1, SOX2 and SOX3. They contain of a N-terminal region followed by the HMG DNA binding domain in addition to a C-terminal activation domain. The SOXB2 group of TFs consisting of SOX14 an SOX21 also contains a N-terminal and HMG domain, however the c-terminal domain acts as a repressor. (Kamachi et al., 2000)

1.1.2 SOX2 is important during CNS development and for adult NSC maintenance

As development progresses SOX2 expression becomes progressively restricted and by 9.5dpc it is observed in the neural tube, sensory placode's, branchial arches, gut endoderm and gonads (Avilion et al., 2003; Wood & Episkopou., 1999). The expression of the other *SoxB1* genes is mainly restricted to the central nervous system (CNS) and overlap with that of *Sox2* (Wood & Episkopou., 1999). More detailed analysis reveals that SOX2 is expressed throughout the neuroepithelium and is maintained post-natally in undifferentiated cells that line the neuroretina, dentate gyrus, rostral migratory stream and subventricular zone. The adult neurogenic regions within the brain that express SOX2 remain proliferative, identified through the incorporation of BrdU. Moreover neurospheres derived from SOX2-

EGFP^{+ve} subventricular zone cells retain the ability to differentiate into all adult CNS cell types (Ellis et al., 2004; Ferri et al., 2004). This suggests that these cells represent neural stem cells (NSCs). In addition SOX2 is also observed in terminally differentiated cells of the cortex, notably in pyramidal neurons (Ferri et al., 2004).

1.1.2.1 SOX2 is required in neural progenitors

Due to the early lethality of *Sox2* null embryos, investigations into the role of SOX2 in the CNS required the generation of hypomorphic and conditional deletion alleles. Moreover, functional redundancy between the three *Soxb1* members often masks, lessens or delays the consequences of deletion of a single gene. Misexpression studies in chick and Xenopus suggested that SOX2 is required to maintain neural progenitor identity (Kishi et al., 2000). In mammals identification of the role of SOX2 in the CNS has primarily focused on regions such as the hippocampus and subventricular zone due to their neurogenic potential. Moreover research has also been conducted in the cortex due to the expression of SOX2 in differentiated cells in this region. SOX2 heterozygosity in mice does not give rise to any obvious CNS phenotype. Consequently compound mutant mice have been used with greater reduction in *Sox2* dosage. Compound $Sox2^{\beta\text{-}Gal/\Delta ENH}$ mutant mice have one allele of *Sox2* replaced by the β-Galactocidase open reading frame and a second allele with a mutated *Sox2* CNS enhancer (*ΔENH*). These express approximately 25-30% of the normal levels of SOX2. These mice have been used to study later roles of SOX2 in the developing CNS (Favaro et al., 2009; Ferri et al., 2004). Morphological CNS defects including a smaller cortex and enlarged third ventricle are seen in $Sox2^{\beta\text{-}Gal/\Delta ENH}$ mice (Ferri et al., 2004). Epileptic spikes are recorded in the hippocampus and cortex and these mice also display a circling phenotype of neurological origin. Defects are also seen at the cellular level including nuclear and cytoplasmic condensation and hyperchromatic cell bodies, markers of neurodegeneration (Ferri et al., 2004). Hippocampal defects are also observed in mice where SOX2 has been conditionally deleted using the *Nestin-Cre* transgene (Favaro et al., 2009). Interestingly both models result in the downregulation of NSC radial glial

markers, Glial Fibrillary Acidic Protein (GFAP) and nestin (Ferri et al., 2004; Favaro et al., 2009). This is also associated with the reduction in the proliferation of these cell types, indicating that SOX2 in NSCs is essential for their maintenance and proliferation. Furthermore, *Sox2* hypomorphic mutant mice display severely impaired neurogenesis as the number of differentiated cells is significantly reduced (Ferri et al., 2004). A similar phenotype is observed in neural retinal progenitors as conditional *Sox2* deletion in these cells using *αP0-CREiresGFP* induces a loss of proliferation and prevents terminal differentiation, through, in part, down-regulation of the direct SOX2 target *Notch1* (Taranova et al., 2006).

1.1.2.2 SOX2 and Sonic Hedgehog interact during CNS development and for NSC maintenance

In the early ventral diencephalon (VD), SOX2, along with SOX3, directly transcriptionally regulates Sonic Hedgehog (SHH) expression, a mechanism essential for maintenance of VD (Zhao et al., 2012). SHH down-regulation in VD midline by a BMP2-TBX2 pathway is required for proper hypothalamic morphogenesis (Manning et al., 2006). It was recently shown that the TFs TBX2 and 3 act to sequestrate SOX2 away from the *Shh* VD enhancer (Zhao et al., 2012), this is necessary to define a SHH negative domain corresponding to the future infundibulum (Trowe et al., 2013). SHH has itself been shown to upregulate SOX9 expression in neurospheres that already express SOX2 (Scott et al., 2010). Significantly this SHH induced upregulation of SOX9 is required for the progression of $SOX2^{+ve}$ neuroepethelial cells into NSCs (Scott et al., 2010). Moreover, GLI2, a downstream mediator of SHH signaling, directly activates SOX2 in embryonic neuroepethelial cells to maintain a proliferative and undifferentiated state (Takanaga et al., 2009). Reciprocally, SOX2 exerts its role for NSC maintenance post-natally at least in part through the upregulation of SHH (Favaro et al., 2009). Altogether these studies show that complex relationships that exist between SOX2 and SHH in the CNS, and that they are important in different contexts.

1.1.2.3 SOXB1 regulation of SIX transcription factors in the CNS

In addition to *Shh*, *Six6* and *Six3* (see below) are also important transcriptional targets of SOX2 in the CNS. Specifically this transcriptional control is demonstrated in the VD where SOX2 and SIX6/3 expression patterns overlap and expression of SIX6/3 is SOXB1 dependent (Lee et al., 2012, Lee et al., 2013). This interaction is also evolutionally conserved as SOX2 can bind the *Six3.2* promoter in Medaka fish, driving its expression in the forebrain (Beccari et al., 2012). In addition to directly promoting SIX expression, SOX2 can also form a complex with another member of the SIX TF family, SIX1. SOX2 and SIX1-EYA physically interact to regulate *Atonal* expression in the mouse cochlea (Ahmed et al., 2012). In the VD SOX2 and SIX3 have been shown to bind the same *Shh* enhancer, SBE2, suggesting transcriptional activation my require co-operative binding between SOX2 and SIX3 (Zhao et al., 2012). These studies indicate SOX2 may be both a direct transcriptional activator, in addition to a transactivational partner of SIX proteins.

In conclusion, SOX2 is required in CNS development, for the maintenance and proliferation of progenitors. SOX2 is also required for direct reprograming of fibroblasts into induced Neural SC (NSC) (Mirakhori et al., 2013), highlighting its importance for both stemness, and neural fate.

1.1.3 SOX proteins interact with partners to regulate transcription

Transcriptional regulation mediated by SOX proteins usually requires the formation of complexes with other TFs (for review see Kondoh & Kamachi., 2010). Members of the different SOX groups interact with a diverse number of partners. *SoxB1*, *C* and *F* TFs primarily bind POU and PAX TFs, such as SOX2-PAX6 complexes in the eye and SOX11-BRN2 in neural progenitors (Kamachi et al., 2001; Tanaka et al., 2004), while *SoxE* TFs can form both heterologous and homologous complexes such as SOX10-MITF in melanocytes and SOX9-SOX9 in chondrocytes (Bridgewater et al., 2003; Ludwig et al., 2004).

One of the best-characterised examples of how SOX proteins regulate expression in combination with a co-factor is the activation of *δ-crystallin* in the developing eye. *δ-crystallin* is one of the first genes expressed in the lens placode and is essential for eye morphogenesis (Kondoh et al., 1987). *δ-crystallin* only becomes expressed following the close apposition of the head ectoderm (future lens) and optic vesicle (future neural retina) (Shinohara., 1976). Inductive signal from the optic vesicle also activates SOX2 expression in the chick head ectoderm prior placode induction (Kamachi et al., 1998). The head ectoderm also expressed PAX6, in a broader domain initially (Kamachi et al., 1998). Co-operative binding of SOX2 and PAX6 on the DC5 *δ-crystallin* enhancer initiates its expression. Moreover co-operativity is essential, as either factor alone does not induce expression (Kamachi et al., 2001). SOX2 and PAX6 transcription factor binding sites (TFBS) lie next to each other on the DC5 enhancer, however the strength of PAX6 binding to the consensus sequence is considerably stronger when SOX2 is present (Kamachi et al., 2001). SOX2-PAX6 complexes can form in the absence of DNA and this interaction appears to only require the HMG domain of SOX2 and paired domain (PD) of PAX6. Nevertheless addition of DNA results in a higher concentration of complex formation. Moreover spacing between the two consensus sites is critical for activity (Kamachi et al., 2001). This demonstrates that both protein-protein and DNA-protein interactions are required for optimal transactivation activity.

Other important partners of SOX2 are members of the paired domain POU TFs. The SOX2-OCT3/4 complex specifically binds to the *Fgf4* promoter to drive its expression in ES cells. In similarity to the SOX2-PAX6 complex, co-operative binding of both proteins is required to drive *Fgf4* expression (Yuan et al., 1995). The importance in the spacer size between either TFBS is also extremely important with the addition of just 2 base pairs (bp) decreasing enhancer activity by a factor of two and 3bp extinguishing it (Ambrosetti et al., 1997). The SOX2-OCT3/4 complex is also responsible for the expression of Undifferentiated Transcription Factor 1 (*Utf1*) in ES cells. In this enhancer, the 3bp spacer between the SOX2 and OCT3/4 TFBS is

however absent. Resolution of crystal structures of the transcription factors on DNA demonstrated that in fact different enhancers mediate the assembly of distinct POU-HMG complexes (Reményi et al., 2003). Complexes formed by members of other SOX groups are also affected by the size of the spacer sequence between either TFBS. *SoxE* proteins such as SOX10 form homodimer complexes that are disrupted by a decrease of 2bp, but unlike the SOX2-OCT3/4 complex on the *Fgf4* enhancer, not affected by a 2bp increase (Peirano et al., 2000). Complex formation between SOXE proteins however is heavily reliant on the N-terminal extension adjacent to the HMG domain. (Peirano et al., 2000). The requirement of co-factors for complex formation with SOX proteins is extremely important to allow highly tissue specific transactivation or repression of downstream targets.

1.1.4 SOX2 is required for development of different organs and mutations in the gene are associated with human diseases

1.1.4.1 Extra-hypothalamo-pituitary axis defects in mice

In mice, *Sox2* mutations are associated with a wide range of extra-hypothalamo-pituitary defects, including in the CNS (discussed above). *Sox2* heterozygous mice are grossly normal (Avilion et al., 2003) but around a third of mutants are lost prior weaning (Kelberman et al., 2006). Male *Sox2* heterozygous mice have reduced fertility, smaller testicles and blocked sperm in the seminiferous tubules, in addition to reduced levels of LH (Avilion et al., 2003; Kelberman et al., 2006). Despite the known role of LH in promoting spermatogenesis, the infertility phenotype is likely a result of impaired sperm maturation at the testes level and not due to reduced pituitary LH, as there is no effect on female fertility.

Mutations in the regulatory regions of *Sox2* that affect expression of the gene in a specific subset of tissues are also associated with developmental defects and have proved useful to study its later functions. Homozygous Yellow Submarine (*ysb*;*ysb*) mice have severe balance and hearing impairment

resulting from a 20 kilobase (Kb) deletion in *Sox2* regulatory regions resulting from transgene insertion (Dong et al., 2002; Kiernan et al., 2005). Similar defects in mice homozygous for the mutation Light coat and circling (LCC) isolated from a random genome mutagenesis screen are also induced by mutation in *Sox2* regulatory regions (Dong et al., 2002; Kiernan et al., 2005). In these mice SOX2 is absent from the prosensory domain of the developing cochlea, resulting in failure, or severe impairment, of hair cell differentiation, inducing balance defects in adult mice (Kiernan et al., 2005).

1.1.4.2 Pituitary abnormalities in *Sox2* heterozygous mice

Sox2 is expressed in the pituitary and hypothalamus throughout development and in adulthood (Fauquier et al., 2008; Kelberman et al., 2006). Two-month-old *Sox2* heterozygous mice have significantly but mildly reduced pituitary levels of GH and LH, coupled with a global growth defect and infertility. Pups, but not adults, also display reduced levels of ACTH suggesting that glucocorticoid deficiency may explain the loss of a proportion of animals (Kelberman et al., 2006). GH levels are decreased at 18.5dpc, prior to post-natal hypothalamic regulation of pituitary secretion suggesting that *Sox2* heterozygosity directly affects pituitary development (Kelberman et al., 2006). At 18.5dpc the pituitary is slightly hypoplastic (Kelberman et al., 2006). Earlier in development, *Sox2* heterozygosity is associated with RP bifurcation, later giving rise to extra clefts observed at 18.5dpc (Kelberman et al., 2006). *Sox3* mutant mice have a similar extra-cleft, reduced hormone levels and pituitary hypoplasia (Rizzoti et al., 2004). SOX3 however is not expressed in RP and this phenotype is a consequence of the loss of *Sox3* in the VD, affecting its morphogenesis and indirectly RP development (Rizzoti et al., 2004). These extra-clefts are unlikely to affect pituitary hormone levels and deficiencies in *Sox3* mutants probably result from hypothalamic defects (Rizzoti et al., 2004). In *Sox2* mutants it is likely that the bifurcations observed result from requirement of the protein in the overlying VD.

1.1.4.3 *SOX2* mutation in humans

In humans, *SOX2* heterozygous mutations preventing or reducing its transcriptional activity are associated with severe eye defects and mental retardation (Fantes et al., 2003), a phenotype more severe than what is observed in *Sox2* heterozygous mice because the gene dosage sensitivity is different, particularly for eye morphogenesis. Indeed, defects observed in humans such as bilateral anophthalmia or severe microphthalmia are only observed in mice if *Sox2* gene dosage is further reduced, in hypomorphic and conditionally deleted mutants (Taranova et al., 2006). However a broad range of human CNS defects including hearing loss (Hagstrom et al., 2005) and hippocampal defects (Sisodiya et al., 2006), are also observed in mice with *Sox2* mutations (Ferri et al., 2004; Kiernan et al., 2005). Moreover, both murine and human *Sox2/SOX2* mutations result in pituitary hypoplasia, however the hormonal deficiencies are different, with gonadotropin reduction being more prominent in humans (Kelberman et al., 2006). This gonadotropin deficiency is likely to originate from hypothalamic defects, as exogenous GnRH administration results in increased LH/FSH production and the number of GnRH neurons is reduced in *Sox2* mutant mice (Jayakody et al., 2012). *In vitro* analysis of the various human mutated SOX2 proteins indicated a loss in DNA binding ability in mutations that resulted in a protein truncation. Loss of the HMG domain resulted in sequestration of SOX2 in the cytoplasm and reduced transcriptional activity was observed when deletions occurred in the transactivation domain (Kelberman et al., 2006).

In summary, SOX2 is crucial for proper embryonic development as it is required in many different contexts. Predominantly expressed in progenitors, SOX2 promotes proliferation and cell fate specification through the binding of co-factors. In humans, heterozygous mutations in *SOX2* are also associated with pleiotropic defects, comprising pituitary defects.

1.2 Pituitary function and regulation

Homeostasis is the physiological status defined as the normal state of internal equilibrium. As the master endocrine gland, the pituitary is key in sustaining homeostasis, acting as the mouthpiece from which the hypothalamus speaks to the rest of the body. Situated at the base of the skull in the sella turcica of the sphenoid bone, the pituitary is central to the neuroendocrine hypothalamic-pituitary axis. Its secretions are controlled by the hypothalamus that directly innervates the neural lobe of the gland, and also secretes neuro-hormones that reach the pituitary via the hypophyseal portal system (Figure 1.2) (for review see Pearson & Placzek., 2013). Subsequently the pituitary releases its own endocrine hormones into the blood, regulating physiological functions such as; growth, metabolism, sexual maturation and function, and skin/coat colour. The pituitary is formed by three lobes; posterior, intermediate and anterior. Each of which secretes its own set of hormones from distinct endocrine cell populations (Figure 1.2).

Also known as the neurohypophysis, the posterior lobe (PL), unlike the other two lobes is an extension of the embryonic neuroepithelium and therefore is linked to the hypothalamus via the pituitary stalk (Figure 1.2). As a consequence, the two hormones that are secreted by the posterior pituitary (PP), oxytocin and vasopressin are synthesised in the hypothalamus and released in the general blood circulation by axonal termini reaching the PP. Specifically oxytocin and vasopressin are produced in the supraoptic and paraventricular nuclei of the hypothalamus where they travel via the axonal projections of magnocellular neurons in the pituitary stalk to the PP (Figure 1.2). This hypothalamic-neurohypophyseal axis regulates in particular parturition (oxytocin) and blood osmolarity (vasopressin).

The intermediate lobe (IL), also known as the neurointermediate lobe, only comprises one type of endocrine cell type, melanotrophs. In mice the IL

forms a defined structure, distinct from the anterior pituitary (AP), however in humans this structure only consists of a thin layer of cells (Figure 1.2). Melanotrophs secrete melanocyte-stimulating hormone (MSH), a type of melanocortin that is proteolytically cleaved from pro-opiomelanocortin (POMC), which stimulates the production of melanin in skin cells. In humans, melanotroph cells are located in particular in the skin.

The anterior lobe (AL) is the largest lobe of the pituitary, and has the same ectodermal embryonic origin as the IL (Figure 1.2, 1.3). The AP comprises six different endocrine cell types, somatotrophs, lactotrophs, thyrotrophs, corticotrophs and gonadotrophs (Figure 1.2). Corticotrophs secrete adrenocorticotropic hormone (ACTH), which like MSH, is proteolytically cleaved from POMC. ACTH secretion is under corticotropin releasing hormone (CRH) control. ACTH acts on the adrenal cortex, stimulating the production of glucocorticoids, mainly cortisol in human and corticosterone in rodents, in response to stress. Pituitary tumors associated with corticotrophs (Cushing's disease) can cause chronic high levels of ACTH, resulting in particular, in extremely high blood pressure. Under the control of thyrotropin-releasing hormone (TRH) secreted from the hypothalamus, thyrotrophs secrete thyroid-stimulating hormone (TSH). TSH acts specifically on the thyroid gland to induce the production and secretion of thyroxine and triiodothyronine, two hormones that increase cellular metabolism. Gonadotrophs secrete two distinct hormones, lutenising hormone (LH), and follicle-stimulating hormone (FSH). Both hormones act in tandem in development, FSH maturing follicles while LH triggers ovulation in females, and induces testosterone production in males. LH/FSH secretion is under the control of hypothalamic gonadotropin-releasing hormone (GnRH). Pituitary gonadotrophins deficiencies are associated with infertility and hypogonadism. Growth hormone (GH) is secreted by somatotrophs in the AP and is involved in different physiological processes, however it is primarily required for post-natal growth. GH is secreted under the control of growth-hormone releasing hormone (GHRH) and somatostatin. Most GH associated diseases originate from pituitary defects,

resulting in dwarfism (deficiency) or acromegaly (excessive secretion). Finally lactotrophs secrete prolactin (PrL), which regulates in particular lactation in pregnant women. The regulation of prolactin secretion is complex and in contrast with other AP hormones the hypothalamus primarily acts to inhibit prolactin release via dopamine secretion.

Figure 1.2: Adult hypothalamic-pituitary axis

Nuclei in the hypothalamus secrete hormones into the hypophyseal portal system that acts on their target endocrine cell in the AP. Five endocrine cell types reside in the anterior lobe; somatotrophs (producing GH), thyrotrophs (producing TSH), corticotrophs (producing ACTH), gonadotrophs (producing LH/FSH), lactotrophs (producing PrL) secrete hormones into pituitary capillaries. The intermediate lobe that divides the posterior lobe from the anterior lobe contains only one cell type, melanotrophs, producing MSH, which also secrete into the pituitary capillaries. Magnocellular neurons send axons from hypothalamic supraoptic and paraventricular nuclei in the hypothalamic-hypophyseal tract, to secrete oxytocin and vasopressin in the posterior lobe where these are collected by capillaries (adapted from Marieb., 2010).

1.3 Rathke's Pouch development

The pituitary embryonic development was first studied in 1839 by German embryologist Martin Rathke, therefore the pituitary anlagen is subsequently referred as Rathke's Pouch (RP). RP gives rise to both the anterior and intermediate lobes while the posterior pituitary and the pituitary stalk, referred as the infundibulum, derive like the hypothalamus from the VD, and are therefore of neurectodermal origin (for review see Pearson & Placzek., 2013). Consequently, the pituitary has a dual embryonic origin and interactions between both territories are required for correct morphogenesis, but also later for proper function of the differentiated hypothalamo-pituitary axis (Figure 1.3) (for review see Davis et al., 2013).

1.3.1 Hypophyseal placode induction

The hypophyseal placode along with the other cranial placode's are ectodermal thickenings that give rise to sense organs and cranial nerves (for review see Baker & Bronner-fraser., 2001). At 8.0dpc, the hypophyseal placode is located rostrally in the anterior neural ridge, directly in contact with the immediately posterior future ventral diencephalon at the most rostral part of the neural plate (Couly & Le Douarin., 1985, 1987; Kouki et al., 2001; Osumi-yamashita et al., 1994). 24h after the formation of the hypophyseal placode, the neural tube bends at the cephalic level (for review see Wilson & Houart., 2004), causing the placode to be displaced posteriorly and ventrally just behind the future telencephalon (Figure 1.3). The hypophyseal placode is also in contact with the anterior end of the foregut endoderm, still overlaid by the future VD (Figure 1.3) (Kawamura & Kikuyama., 1995).

1.3.2 Rathke's pouch induction

RP forms as the hypophyseal placode extends toward the VD, by 9dpc. RP formation requires the presence of the overlying VD and the infundibulum, that starts to form a funnel shaped structure as RP develops (Figure 1.3). Physical ablation of the VD in chick prevents formation of RP (Kawamura & Kikuyama., 1998). Conversely early ectopic formation of RP at the 10-somite stage in chicks can be induced in explants using VD and head ectoderm (Gleiberman et al., 1999). This shows that head ectoderm is competent to form RP and requires contact/signals from the VD to do so. In mammals, the requirement of VD and infundibulum for proper RP development is highlighted in particular by *Nkx2.1* null phenotype. *Nkx2.1* is a TF expressed at the midline in the VD, but not in RP. *Nkx2.1* null embryos have no VD midline and, as a consequence, no pituitary (Kimura et al., 1996). The early physical ablation studies indicated that the VD and infundibulum might secrete factors required for the development of RP. More recent studies have identified a number of factors expressed and secreted in the VD that help control RP development (Brinkmeier et al., 2003; Brinkmeier et al., 2007; Davis & Camper., 2007; Ericson et al., 1998; Treier et al., 1998).

Figure 1.3: Development of RP and adult pituitary gland

Formation of the pituitary in the embryo, developmental stages (in days post-coitum, (dpc)) as indicated. RP (pink) forms from the hypophyseal placode following invagination of the oral ectoderm ventrally at 8.5dpc. By 12.5dpc the infundibulum has evaginated from the overlying neurectoderm and lies adjacent the dorsal region or RP which will form the future IL. But 18.5dpc/adult the anterior pituitary has expanded, while the IL forms a layer surrounding the PL, which has extended from the hypothalamus via the pituitary stalk. 8.5dpc – 12.5dpc are orientated sagittally, adult is orientated coronally (from Rizzoti & Lovell-Badge., 2011).

1.3.3 Ventral Diencephalon regulation of Rathke's Pouch formation

1.3.3.1 BMP4 is required for induction and maintenance of Rathke's Pouch

Bone morphogenetic proteins (BMP's), are secreted growth factors that belong to the TGFβ superfamily. Binding to serine/threonine kinase receptors BMPs induce transcription of target genes through the Smad signalling pathway (for review see Chen et al., 2004). Of the 20 known BMPs only BMP4 is expressed in the VD, with its expression first observed at 8.5dpc, just after the onset of hypophyseal placode formation at 8.0dpc (Ericson et al., 1998). BMP4 secretion from the VD is essential in RP induction as deletion of *Bmp4* result in a failure to form RP (Takuma et al., 1998). Similarly to what is observed in *Bmp4* null mice, ectopic expression of *noggin* (secreted BMP antagonist) arrests RP development (Treier et al., 1998). Conversely, *Noggin* null embryos have an expanded area of BMP4 expression in the VD and display a variable phenotype, from induction of a rostral shift in RP formation to formation of a second RP (Davis & Camper.,

2007). The expanded area of BMP4 expression in *noggin* null embryos induces a rostral shift in the expression domain of the secreted factor SHH and the transcription factor SIX6 (see below) (Davis & Camper., 2007).

In addition to participating in RP induction, BMP4 is likely involved in progenitor maintenance and proliferation. This regulation is likely to be exerted at several levels. First, conditional deletion of the *Bmp* receptor *Bpmr1a,* expressed in RP, prevents expression of the TF ISL1 in RP (see below), indicating that BMPs modulates TF expression in RP (Davis and Camper., 2007). Second, BMP4 can also regulate expression of the fibroblast growth factor member FGF10 in VD (see below). FGF10 is normally expressed in an overlapping pattern with BMP4 in the VD, however, increased BMP4 expression in *noggin* null embryos results in down-regulation of FGF10 expression in the VD, which in turns affect expression of FGF dependent targets in VD (see below, Davis and Camper., 2007). The influence of BMP4 expression on other factors expressed in the VD highlights the balance that must be achieved between BMP and FGF in order to promote correct RP development.

1.3.3.2 FGFs control Rathke's Pouch progenitor proliferation

Fibroblast Growth Factors (FGF's), FGF8, FGF10 and FGF18 are members of a large family of secreted molecules that activate tyrosine kinase receptors (for review see Ornitz & Itoh., 2001). All three are expressed in the VD slightly later than BMP4, from 9.5dpc, while only one receptor, FGFr2IIIb that binds FGF10, is expressed in RP (Ericson et al., 1998; De Moerlooze et al., 2000; Treier et al., 2001; Treier et al., 1998). Interestingly *Fgfr2IIIb* null mice can only form a small, undeveloped RP. *Fgf10* null mice closely mimic this phenotype, indicating the importance of FGF signalling in RP development (De Moerlooze et al., 2000; Ohuchi et al., 2000).

FGF8 has been shown to bind to FGFR4 and FGFRc splice forms, however none of these as yet have been reported in RP (Zhang et al., 2006). It is

suggested that it can interact with *Fgfr2IIIb,* of which transcripts are observed in the dorsal region of RP, adjacent to the FGF8 signalling from the VD (Takuma et al., 1998). RP is competent to respond to FGF8 as pMAPK, an intracellular mediator of FGF signalling is found in its active, phosphorylated from, in RP between 10.5dpc and 14.5dpc (Davis & Camper et al., 2007). Significantly, pMAPK is observed forming a gradient in RP at 10.5dpc, which is higher in the dorsal region, close to the source of FGF8 (Davis et al., 2011). This gradient is downregulated following cell cycle exit at 11.5dpc, suggesting that it promotes early progenitor proliferation (Davis et al., 2011). The loss of FGF8 in the VD of *Nkx2.1* null mice is suggested to result in failure of RP development, as BMP4 is initially maintained in these mutants as RP is induced, but fails to progress further (Takuma et al., 1998). The role of FGF8 is likely to induce progenitor cell proliferation through the upregulation of the TFs LHX3 and LHX4 (see below) as both of these factors are downregulated in *Nkx2.1* null mice (Takuma et al., 1998). But its role is more complex because it can also induce the downregulation of the TF ISL1 (see below) in pituitary explants (Ericson et al., 1998, see below). *In vivo*, restriction of ISL1 to the ventral region of RP is associated with loss of proliferation and onset of thyrotroph and corticotroph differentiation (Norlin et al., 2000). Consequently the role of FGF signalling in the VD is to promote proliferation in the dorsal region of RP and preventing early differentiation.

1.3.3.3 Wnt/β-catenin pathway controls VD patterning and Rathke's Pouch proliferation

The Wingless-type (Wnt) family of signalling molecules is involved in multiple developmental processes (for review see Amerongen & Nusse., 2009). Wnt ligands bind to the frizzled family of seven transmembrane domain receptors in addition to co-receptors of the LRP family. The canonical Wnt pathway acts via cytoplasmic β-catenin. In the absence of Wnt, β-catenin is targeted for ubiquitination through complex formation with the destruction complex. Conversely in the presence of Wnt, β-catenin

can shuttle into the nucleus to activate transcription (for review see Amerongen & Nusse., 2009).

Two Wnt ligands are expressed during RP development. *Wnt5A* is expressed throughout the VD midline from 9.5dpc and is maintained in the infundibulum until 14.5dpc (Potok et al., 2008). *Wnt5A* deletion results in alteration of FGF8 and BMP4 expression pattern boundaries and in consequence RP bifurcations (Cha et al., 2004; Potok et al., 2008). Wnt4 is similarly expressed in the VD at 9.5dpc, however it is also present in RP (Treier et al., 1998). *Wnt4* mutant mice only display mild defects in endocrine cell expansion, likely a result of loss of Wnt4 in the RP, and not VD (Treier et al., 1998; Potok et al., 2008). TCF4, a downstream TF activated by the canonical Wnt pathway is expressed in the VD and RP. Like *Wnt5A* mutants, *Tcf4* null mice have expanded BMP and FGF expression domains in the VD, likely contributing to the mild hypoplasia observed in these mice (Brinkmeier et al., 2003, Brinkmeier et al., 2007).

As described above the canonical Wnt pathway acts in particular through intracellular β-catenin signalling. Initial experiments, including in particular treatment of embryos with the β-catenin activator lithium chloride, that stabilises β-catenin, suggested that Wnt signalling was in involved induction of expression of *Pitx2* (see below), a TF expressed in RP progenitors that in turn promoted progenitor proliferation through upregulation of *Cyclin D1* (Kioussi et al., 2002). Subsequently, β-catenin gain and loss of function experiments revealed that the protein may be involved in later determination events and that it interacted with the TF PROP1 (see below, Olson et al., 2006). In a more recent study, however, different results were obtained by inducing early expression of a constitutively active form of β-catenin (Gaston-Massuet et al., 2011). Gain of function resulted here in an increase in the progenitor pool and subsequent development of tumors displaying features of craniopharyngiomas, human pituitary paediatric tumours associated with β-catenin mutations (Gaston-Massuet et al., 2011). All together these studies suggest Wnt signalling is necessary for proper

patterning of VD, which indirectly affects RP development, and also that it is required within RP for proliferation of progenitors.

1.3.4 Oral ectoderm regulation of Rathke's Pouch formation

1.3.4.1 BMP2 dependent ventral Rathke's Pouch patterning

BMP2 is expressed in the oral ectoderm, the ventral region of RP at 10.5dpc and also in the mesenchyme surrounding RP (Ericson et al., 1998; Treier et al., 1998; Davis et al., 2011). By 11.5dpc BMP2 expression expands throughout RP and the active, phosphorylated form of SMAD, transducer of BPM signaling, follows this pattern (Davis et al., 2011). By 14.5dpc both BMP2 and pSMAD are downregulated in the majority of cells in RP (Davis et al., 2011). As mentioned above, BMP signaling is involved in ISL1 activation in RP and BMP2 may induce its ventral expression at 10.5dpc (Davis and Camper., 2007). Finally, ectopic overexpression and maintenance of BMP2 expression result in failure to produce terminally differentiated somatotrophs, thyrotrophs, lactotrophs and gonadotrophs (Treier et al., 1998). In conclusion, BMP2 within and around the pouch may be necessary at 10.5dpc for emergence of the first cell type to differentiate, rostral-tip thyrotrophs, trough ISL1 activation, but its activity must be down-regulated for terminal differentiation to proceed.

1.3.4.2 SHH dependent Rathke's Pouch proliferation

Sonic Hedgehog (SHH) and its mammalian paralogues, Indian Hedgehog and Desert Hedgehog are classical morphogens involved in patterning and cell fate decisions (for review see Ribes & Briscoe., 2009). SHH binds the Patched receptor on the cell membrane, inducing activation of the Gli family of TFs. *Gli1, Gli2* and *Gli3* are all expressed in RP demonstrating its capacity to respond to SHH signals (Hui et al., 1994). SHH is expressed in the VD where it is transcriptionally regulated by SOXB1 proteins (see above), and it is also present throughout the oral ectoderm, but excluded from RP (Treier

et al., 2001). Ectopic overexpression of SHH in RP using the α-GSU promoter, α-glycoprotein sub-unit, first marker of endocrine differentiation appearing ventrally at 11.5dpc, results in expansion of thyrotrophs and gonadotrophs ventrally (Treier et al., 2001). Conversely, overexpression of SHH antagonist, Hip, results in pituitary hypoplasia (Treier et al., 2001). This suggests that SHH may induce progenitor proliferation.

More recently, conditional deletion of *Gli2* in RP was shown to result in a reduction of progenitor proliferation in the ventral region of RP at 10.5dpc (Wang et al., 2010). Conversely constitutive activation of the SHH pathway in RP results in enhanced proliferation in the dorsal region of RP (Wang et al., 2010). This suggests that the dorsal region of RP proliferates in response to SHH from the VD. In mutant pituitaries, cell determination is not affected (Wang et al., 2010). Therefore SHH signaling from the VD and oral ectoderm may promote progenitor proliferation in the early RP but not cell fate specification.

Midline defects in humans including holoprosencephaly (HPE) and Septo-optic Dysplasia (SOD) are associated in particular with mutations in *SHH* (Gorbenko et al., 2013). These midline defects comprise a failure to fuse the basisphenoid bone, upon which the pituitary is located (Figure 1.3). This defect is also seen in mutations in genes encoding for proteins involved in the intracellular transduction of SHH signaling (Khonsari et al., 2013). Failure to fuse the sphenoid bone is associated with a failure of RP to detach form the underlying oral ectoderm (Khonsari et al., 2013). This suggests that ventral SHH signaling in the oral ectoderm is also essential for correct sphenoid bone formation, which indirectly affects pituitary morphogenesis.

1.3.5. Notch Signaling in Rathke's Pouch

The Notch pathway is initiated by the binding of a transmembrane ligand to a Notch receptor located on an adjacent cell. Subsequent cleavage of the Notch intracellular domain allows it to activate transcription of downstream

target genes such as *Hes1* and *Hey1* (for review see Kopan & Ilagan., 2009). In RP two ligands of the Notch pathway, Delta-like 1 and Jagged1, in addition to receptors Notch2 and Notch3 are expressed by 12.5dpc (Zhu et al., 2006). Hes1 is expressed throughout RP at 10.5dpc, however it is downregulated by 16.5dpc (Raetzman et al., 2007). Functionally HES1 promotes progenitor proliferation in the early RP, and its overexpression in differentiating gonadotrophs and thyrotrophs cells prevents terminal differentiation (Monahan et al., 2009; Raetzman et al., 2007). Similarly constitutive Notch overexpression in the POMC lineage prevents corticotroph and melanotroph differentiation (Goldberg et al., 2011). This suggests that Notch signaling in the early RP promotes progenitor proliferation and prevents early differentiation.

The activities of BMP4, FGFs, Wnt5A and SHH in the ventral diencephalon must be fine-tuned to allow for the correct development of RP. Alterations in their respective pattern of expression or activity can result in abnormal pituitary morphogenesis but they do not directly affect endocrine cell specification as endocrine differentiation still occur despite impaired VD patterning and signaling in mutants. Ventrally SHH and BMP2 are also important for correct RP formation. Within the pouch BMP2, Wnt and Notch signaling are involved in control of progenitor proliferation and presumably participate in cell determination events. These factors work in a non-cell autonomous fashion to regulate RP induction and development, and allow expression of a cascade of TFs that are initially involved in progenitor maintenance, and later allow emergence of the different endocrine cell lineages.

1.4 Transcription factors required for Rathke's Pouch progenitor maintenance and proliferation

1.4.1 SIX6/3

The *Six* genes are evolutionarily derived from the *Sine Oculis* homeobox group of TFs. In *Drosophila Melanogaster* where they were first identified as inducing eye formation, there are three *Six* subgroups, *SO, Opitx* and *Dsix4,* however a genome duplication event in evolution has resulted in six *Six* genes in vertebrates: *Six1/2* (*SO*), *Six6/3* (*Opitx*) and *Six4/5* (*DSix4*). In addition to Drosophila and humans the SIX genes have been highly conserved throughout evolution, with at least one subgroup found in 40 invertebrate genomes and 50 vertebrate genomes (for review see Kumar., 2009). At the protein level the SIX TFs consist of DNA binding homeodomain (HD), sharing at least 63% homology across the subgroups and a protein-protein binding SIX domain (SD). Significantly the SD can bind both co-activators such as EYA and induce ectopic eye formation or co-repressors DACH and Groucho to inhibit eye formation (Li et al., 2003; Pignoni et al., 1997; Zhu et al., 2002).

Of the six SIX TFs found in vertebrates, only SIX Homeobox 6 (SIX6) and SIX Homeobox 3 (SIX3) are expressed in the pituitary. As paralogues, SIX6 and SIX3 often share overlapping patterns of expression and are both observed in the invaginating RP at 10.5dpc (Jean et a., 1999; Oliver et al., 1995). In addition both are expressed in the overlying VD, infundibulum and presumptive hypothalamus (Jean et al., 1999; Oliver et al., 1995). Both *Six6* and *Six3* null mice display similar phenotypes, including small or absent eyes. In *Six3* null embryos, forebrain defects are so severe that formation of RP is prevented. However, conditional deletion of the gene in the brain or pituitary leads to post-natal growth defects and early death suggesting that SIX3 is required both within the VD and RP for normal pituitary function (Gaston-Massuet et al., 2008). *Six6* null mice display a hypoplastic pituitary (Li et al., 2002) and this is likely explained by a reduction in proliferation

observed in RP (Li et al., 2002). This is potentially caused by direct repression of cyclin dependent kinase inhibitor *p27kip1,* as *in vitro* assays demonstrate the ability of SIX6 to directly bind the *p27kip1* promoter and repress its activity in αT3 pituitary cells (Li et al., 2002). Conversely, in *Tcf4* null mice, recruitment of a larger domain of oral ectoderm results in expansion of SIX6 expression and is associated with increased proliferation (Brinkmeier et al., 2007). Human mutations in *SIX6/3* are associated with mid-line defects including bilateral anophthalmia and panhypopituitarism reminiscent of the phenotype observed in *Six6* null mice (Gallardo et al., 1999). These studies suggest SIX6/3 play an important role in RP development and are involved in the regulation of RP progenitor proliferation.

1.4.2 LHX3/4

Lhx3 and *Lhx4* are members of the LIM-homeodomain group of TFs (for review see Mullen et al., 2007). LHX3 and LHX4 contain a DNA binding HD domain and a protein-protein binding LIM domain. Both are first expressed in RP at 9.5dpc and their function is initially redundant (Sheng et al., 1996). *Ex vivo* experiments suggest that their expression is induced by FGFs, starting to be secreted from the VD at the same stage (Ericson et al., 1998). *In vivo*, absence of FGF8 in *Nkx2.1* null mutants VD correlates with a failure to activate both LHX3 and LHX4 expression (Takuma et al., 1998) and a failure to maintain RP, also observed in *Lhx3;Lhx4* double mutants (Sheng et al., 1997). *Lhx3* expression can also be directly regulated by ISL1 and PITX1 (see below) as both have the capacity to bind a 180bp upstream enhancer, driving *Lhx3* expression in the pituitary and spinal cord (Mullen et al., 2012).

LHX3 is required at two different phases. It is initially involved in maintaining the progenitor cell pool early in development. *Lhx3* null mice can form an RP, however its expansion is arrested at 12.5dpc. This is associated with downregulation in ISL1 and HESX1 and an increase in apoptosis (Sheng et al., 1996). Thereafter, at 16.5dpc in wild type embryos,

as LHX3 is expressed ubiquitously, homozygous deletion results in failure to express the transcription factor PIT1 and consequently a severe reduction in somatotrophs, lactotrophs and thyrotrophs (Sheng et al., 1996). Additional analysis of *Lhx3* null mice also revealed downregulation in TPIT and NeuroD1, coupled with further increase in apoptosis and a loss of Notch2 signaling (Ellsworth et al., 2008). LHX3 can also directly activate transcription of *Pit1*, TSH, FSH and *Foxl2* (Bach et al., 1995; Ellsworth et al., 2006; Sloop et al., 1999; West et al., 2004). Mutations in *LHX3* in humans are associated with Combined Pituitary Hormone Deficiency (CPHD) where only ACTH is unaffected (Netchine et al., 2000).

In contrast with LHX3, LHX4 is downregulated during late embryogenesis (Sheng et al., 1997). *Lhx4* null mice die shortly after birth, displaying a hypoplastic pituitary, however in contrast with *Lhx3* null mice, all five AP endocrine cells form, highlighting the specific and exclusive role of LHX3 in regulating endocrine differentiation (Sheng et al., 1997). LHX4 may be required along with PROP1 (see below) for the transcriptional activation of *Lhx3* expression, as *Lhx4*$^{-/-}$*;Prop1*$^{-/-}$ mice, in contrast with single mutants, do not express LHX3 (Raetzman et al., 2002). In humans mutations in *LHX4* also result in CPHD (Machinis et al., 2001).

1.4.3 ISL1

Insulin gene enhancer protein 1 (*Isl1*) is also a member of the LIM-homeodomain group of TFs (Bhati et al., 2008). ISL1 is expressed throughout the oral ectoderm at 8.5dpc, becoming restricted to RP at 9.5dpc (Ericson et al., 1998). It is then further restricted to the ventral region of RP between 10.5-11.5dpc (Ericson et al., 1998). The ventral restriction of ISL1 is suggested to be due by the negative action of FGFs secreted from VD as exposure of RP explants to a FGF8 coated bead results in downregulation of its expression (Ericson et al., 1998). This regulation is temporally specific however as once ISL1 becomes restricted to the ventral region of RP it no longer responds to FGF8 signaling. As mentioned above, BMPs are involved

in activation of its expression (Davis and Camper., 2007). Upon ventral restriction, ISL1^{+ve} cells begin to express αGSU and become fully differentiated PIT1 independent rostral-tip thyrotrophs 24h00 later (Ericson et al., 1998). Later in development, ISL1, in combination with GATA Binding Protein 2 (GATA2) and LHX3, can bind the gonadotropin releasing hormone receptor (GnRHr) promoter and may be involved in regulating its expression (Schang et al., 2013).

Isl1 null embryos die at 10.0dpc however they can form a RP, albeit a hypoplastic one, suggesting the protein is initially required for progenitor maintenance (Takuma et al., 1998). *Lhx3* null mice fail to maintain *Isl1* expression in RP at 12.5dpc but then display a dorsal ectopic expression of the gene between 16.5-18.5dpc (Ellsworth et al., 2008). This highlights a complex transcriptional regulation, probably linked to its expression in different cell types, initially in progenitors and later in differentiated endocrine cells.

1.4.4 PITX1/2

Paired-like homeodomain 1 and 2 (*Pitx1/2*) are members of the bicoid class of homeodomain proteins and share a degree of functional redundancy. The homozygous deletion of either gene does not affect the expression of LHX3, while the combined double mutation results in its complete downregulation with allelic series displaying intermediate phenotypes (Charles et al., 2005; Suh et al., 2002). The requirement for each TF in RP development and pituitary function is however different.

PITX1 is expressed throughout the oral ectoderm from 8.0dpc, becoming restricted to RP at 9.5dpc and thereafter maintained in all terminally differentiated endocrine cell types (Lanctôt et al., 1997). No abnormalities are initially observed in the pituitary of *Pitx1* null embryos, likely because of a functional redundancy with PITX2 (Charles et al., 2005; Gage et al., 1999). Later in development PITX1 is involved in the transcriptional regulation of a

number of genes encoding for hormones, including; Prl, GH, FSH and POMC (Lamolet et al., 2001; Szeto et al., 1996; Trembley et al., 1998). Moreover PITX1 has also been shown to drive the expression of lineage markers *Pit1* and *Sf1* (Szeto et al., 1996; Tremblay et al., 1998). In *Pitx1* null mice, there is a decrease in the number of gonadotrophs and thyrotrophs, and consequently lower levels of LH and TSH levels, probably reflecting the requirement for PITX1 in transcriptional regulation in endocrine cells (Gage et al., 1999).

PITX2 expression is first detected in RP at 8.5dpc, later becoming ubiquitous in the gland. Postnatally, expression is mainly restricted to gonadotrophs and thyrotrophs (Gage et al., 1997). Early on in development PITX2 appears to play an important role in RP formation and maintenance, as development is arrested at 12.5dpc in *Pitx2* null mice (Gage et al., 1999). PITX2 is an important regulator of progenitor proliferation and was shown to promote cell cycle progression through the activation of *Cyclin D1* and *D2* (Kioussi et al., 2002). Progenitor proliferation can be further increased by the upregulation of PITX2 via activation of Wnt/βcatenin signaling, suggesting a role for the Wnt pathway in promoting PITX2 expression (Kioussi et al., 2002). Hypomorphic *Pitx2* mutants also display reduced expression of LH, FSH, GH and TSH (Suh et al., 2002). In conclusion, PITX2 has an earlier, and more preponderant role than PITX1, in progenitor maintenance and proliferation and it may play a later role in promoting endocrine cell differentiation.

1.4.5 HESX1

Homeobox expressed in ES cells 1 (HESX1) is a member of the paired-like class of homeodomain TFs functioning as a repressor, by co-binding in particular Transducin-Like Enhancer of Split 1 (TLE1) (a mammalian Groucho family member) (Dasen et al., 2001). HESX1 is first expressed in the anterior neural ridge where the hypophyseal placode will develop, and also in the rostral neural plate that will form the forebrain (Paul Thomas &

Beddington., 1996; Thomas et al., 1995). Its expression might be regulated by the Lim homeodomain transcription factors LHX1 and LHX3 (Chou et al., 2006). LHX1 bind enhancer regions 5' of *Hesx1* to drive expression in the forebrain, while LHX3 binds an additional 3' enhancer to activate transcription in RP, probably along with PITX2 and GATA2/3 (Chou et al., 2006). HESX1 is essential for correct development of the forebrain, and *Hesx1* null mice display post-natal lethality likely caused by CNS defects comprising a reduced prosencephalon, anophthalmia, and pituitary dysplasia (Dattani et al., 1998). Loss of HESX1 in the anterior neurectoderm results in posteriorisation, possibly due to ectopic activation of the Wnt/βcatenin pathway (Andoniadou et al., 2007; Martinez-barbera et al., 2000). As development progresses HESX1 is restricted to RP at 9.5dpc where it is maintained until 13.5dpc (Hermesz et al., 1996). The early development of RP in *Hesx1* null embryos is characterised by a variable phenotype comprising multiple clefts, over proliferation and often misplacement of the gland in the naso-pharyngeal cavity, aspects of which are likely to be explained by loss of the gene in VD (Dasen et al., 2001). Later endocrine cell differentiation occurs normally, but with increased numbers (Dasen et al., 2001). After birth, hypoplasia of the pituitary is observed and this is likely the result of impaired hypothalamic control. In humans, *HESX1* mutations are associated with Septo-optic Dysplasia (SOD), a syndrome whose characteristics closely resemble those displayed by *Hesx1* null mice (Dattani et al., 1998).

An important role of the HESX1/TLE1 complex is to inhibit expression of the TF PROP1 (see below) (Dasen et al., 2001). PROP1 is required for emergence of endocrine cell lineages and the switch of expression between HESX1 and PROP1 is crucial for correct development of the gland (Dasen et al., 2001). *Hesx1* downregulation is likely to be mediated through the Wnt/β-catenin pathway as a β-catenin/PROP1 complex was shown to inhibit *Hesx1* expression (Olson et al., 2006).

The complex relation between HESX1 and other early TFs was further highlighted by the phenotype of compound *Six3*;*Hesx1* mutants. While *Six3* and *Hesx1* mutants display similar forebrain defects, *Six3*$^{-/-}$ defects are more severe and RP doesn't form, probably due to VD abnormalities. Intriguingly, *Six3*$^{+/-}$;*Hesx1*$^{Cre/+}$ (Cre replaces *Hesx1* so *Hesx1*^{Cre} allele is effectively a null) double mutant embryos display increased early progenitor proliferation and multiple clefts, comparable to *Hesx1*$^{-/-}$ mutants. This is suggested to result from increased Wnt signaling, promoting progenitor proliferation (Gaston-Massuet et al., 2008). These studies highlight the important yet complex role HESX1 plays in pituitary development, as it acts in the forebrain and RP.

1.4.6 PROP1

Paired-like homeobox 1 (*Prop1*) is the earliest gene to be exclusively expressed in RP. It starts to be expressed at 10.0dpc and is downregulated after 12.0dpc, however it is maintained in some of the cells that line the lumen in the adult pituitary (Sornson et al., 1996; Yako et al., 2011). PROP1 is initially present throughout RP, along with SOX2 (see below) (Yoshida et al., 2009). Notch signaling induces its expression in RP (Zhu et al, 2006). Later PROP1 is maintained in a few SOX2^{+ve} SC postnatally (Yako et al., 2011). *Prop1* null, and the naturally occurring Ames Dwarf mutants (mutation inducing low DNA binding activity), both display reduction in somatotrophs, lactotrophs, thyrotrophs and gonadotrophs (Sornson et al., 1996). During development, progenitors fail to leave the dorsal progenitor zone and to differentiate, resulting in retention of cells in the periluminal region of RP (Ward et al., 2006). Another Notch target, Hes1, may be involved in regulating these progenitor cells movement and differentiation, as suggested by the phenotype of *Hes1;Prop1* double mutants (Himes et al., 2009). An important target of PROP1 is the TF *Pit1*, required for the emergence of several endocrine cell types (Andersen, 1995, see below). Ames Dwarf mice also show a complete loss of PIT1 expression while HESX1 expression is retained beyond its normal temporal limit (Andersen et al., 1995; Olson et al., 2006). Only 9.7% of cells in RP co-express both PROP1

and PIT1 at its highest point at 16.5dpc in *Rattus Rattus* RP (Yoshida et al., 2009) suggesting that the interaction is very transient. Importantly PROP1 acts as both a repressor and activator simultaneously. Forming a complex with β-catenin, PROP1 can inhibit *Hesx1* expression and promote *Pit1* expression, promoting lineage specification (Olson et al., 2006).

Mirroring the Ames Dwarf mouse model, human mutations in *PROP1* are associated with CPHD and more specifically reductions in GH, TSH, and PrL. This is in addition to failure to enter puberty, likely due to a reduction in gonadotropin secretion (Wu et al., 1998). In conclusion PROP1 is an important factor underlining progression from proliferating progenitors to differentiating cells. In RP, this transition is associated with movements of cells that PROP1 regulates, from a dorsal proliferative zone to a ventral region where the future AL takes shape.

1.4.7 SOX2

SOX2 is expressed ubiquitously in RP from at least 11.5dpc and in the overlying VD, comprising the infundibulum both in mice and humans (Fauquier et al., 2008; Kelberman et al., 2008; Wood & Episkopou, 1999). Due to its wide expression pattern, study of its role within RP has relied on the development of a conditional deletion approach (this thesis). As development progresses, SOX2 becomes progressively restricted to the dorsal region of RP containing progenitors for both AL and IL. It is maintained post-natally in a population of cells that line the pituitary cleft, which is a remnant of RP lumen (Figure 1.4 and 1.5) (Fauquier et al., 2008). $SOX2^{+ve}$ cells are also observed scattered in the AP parenchyma and are $hormone^{-ve}$ (Figure 1.5) (Fauquier et al., 2008). $SOX2^{+ve}$ cells are highly proliferative at 12.5dpc, however this declines throughout development and the cells are mostly quiescent in adults. Recent studies have shown that the number of $SOX2^{+ve}$ cells is very high during the neonatal growth phase of the rodent pituitary, and decreases later in life (Gremeaux et al., 2012). It is now clearly established that $SOX2^{+ve}$ cells are adult pituitary stem cells (SCs) (see

below). In humans, heterozygous mutations in *SOX2* are associated with bilateral anophthalmia, severe microphthalmia and hypopituitarism (Fantes et al., 2003; Kelberman et al., 2006, see above).

1.5 Endocrine cell differentiation

Following expansion of RP progenitors, endocrine cell specification and differentiation take place. There are three endocrine lineages, characterised by the expression of a specific TFs, the earliest that committed cells express. The largest of the three lineages is the PIT1 lineage (Figure 1.4). This gives rise to somatotrophs, lactotrophs and thyrotrophs (excluding rostral-tip, PIT1 independent thyrotrophs, which form a transient population disappearing a birth). The SF1 population exclusively forms gonadotrophs. Finally, the TPIT lineage is comprised of corticotrophs in the AL and melanotrophs in the IL.

Birthdating studies have demonstrated that AP precursors exit the cell cycle concurrently between 11.5dpc and 13.5dpc (Davis et al., 2011). Within this time frame the different endocrine cell populations peak exit time is slightly different; gonadotroph and thyrotroph progenitors exit the cell cycle between 11.5dpc – 12.5pc, while corticotroph and somatotroph progenitors stop proliferating 24h00 later between 12.5dpc – 13.5dpc. Melanotroph precursors are the latest to exit the cell cycle 12.5dpc – 14.5dpc (Davis et al., 2011). Cell cycle exit is associated with expression of cyclin dependent kinase inhibitor (CDKI) p57kip1 (Bilodeau et al., 2009). p57kip1^{+ve} cells are situated below the dorsal progenitors and above ventral differentiated cells and mark an intermediary, non-cycling, undifferentiated population. Terminal differentiation however is associated with the up-regulation, initially in a ventral territory then expansion of another CDKI, p27kip1, preventing cells from re-entering the cell cycle (Bilodeau et al., 2009). In the developing gland, differentiated endocrine cell type form homotypic networks. In the adults, these networks allow efficient and coordinated hormonal release (Figure 1.4) (for review see Mollard et al., 2012).

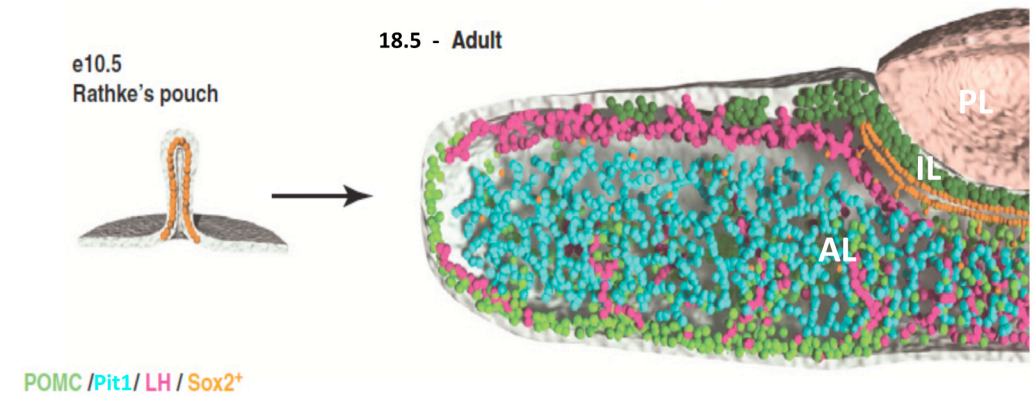


Figure 1.4: SOX2 and endocrine cell expression in RP and pituitary

SOX2 is ubiquitously expressed in RP at 10.5dpc. In the adult pituitary the RP lumen persists as the pituitary cleft and is lined by the SOX2^{+ve} population, as observed in the embryo. Corticotrophs (ACTH) and Melanotrophs (MSH) of the POMC lineage, somatotrophs (GH) of the Pit1 lineage and gonadotrophs (LH) of the SF1 lineage form networks in the anterior lobe (AL) and intermediate lobe (IL) of the 18.5dpc and adult gland. Conversely the two other members of the Pit1 lineage, thyrotrophs (TSH) and lactotrophs (PrL) do not form networks. No endocrine cells reside in the posterior lobe (PL). Schematic represents one half of the adult pituitary in a coronal orientation (adapted from Mollard et al., 2012).

1.5.1 PIT1 lineage

First expressed in RP at 13.5dpc POU class 1 Homeobox 1 (*Pou1f1*) also known as Pituitary Specific Transcription Factor 1 (*Pit1)*, is induced by PROP1 (Andersen et al., 1995) and marks a population of cells that will become somatotrophs, lactotrophs and thyrotrophs (Figure 1.4) (Bodner et al., 1988). The phenotype of two naturally occurring mutations in *Pit1* in mice, *Snell dwarf* (point mutation in DNA binding domain) and *Jackson dwarf* (no expression following chromosomal rearrangement) have highlighted the requirement for PIT1 in these three cell types (Li et al., 1990). Interestingly the reduction in somatotrophs, thyrotrophs and lactotrophs observed in both strains and also engineered null mutants, does not occur until after birth (Li et al., 1990; Ward et al., 2006). Post-natal hypoplasia is associated with reduction in proliferation and apoptosis, indicating that PIT1 is not required for emergence of these cell types but for their post-natal expansion (Ward et al., 2006). The aetiology of this phenotype is still unknown. Consistent with the phenotypes observed in

Snell and *Jackson dwarf* mice human mutation in *PIT1* result in reductions in PrL, TSH and GH (Pfäffle et al., 1992; Radovick et al., 1992).

PIT1, along with different co-factors, regulates the expression of the hormones characterising the three cell types of the PIT1 lineage. In lactotrophs, PIT1 binds to a distal PrL enhancer to drive PrL expression *in vitro*, however addition of estradiol (E2) greatly enhances the level of expression (Day et al., 1990). This interaction may play a role in pregnancy where Prl levels increase. In somatotrophs, PIT1 can bind to the GH promoter to drive its expression and co-binding with zinc finger protein Zn-15 greatly increases activity, while mutations in this region dramatically reduce GH expression highlighting the functionality of this interaction (Lipkin et al., 1993). Similarly PIT1 and the TF GATA2 co-operate to drive expression of TSH (Gordon et al., 2002).

1.5.2 SF1 lineage

Unlike the two other lineages discussed in this section the Steroidogenic Factor 1 (*Sf1*) lineage give rise to only one endocrine cell type, gonadotrophs (Figure 1.4). Also called Nuclear Receptor 5a1 (*Nr51a)*, SF1 is also expressed in hypothalamus where it regulates GnRH expression, the adrenal gland and gonads where it up-regulates the expression of *Sox9* during testis development (for a review of the latter see Sekido & Lovell-badge., 2009). In RP, SF1 is first observed in future gonadotrophs at 13.5dpc, where it is maintained (Ingraham et al., 1994). Homozygous deletion of *Sf1* results in disrupted gonad development in addition to sex reversal, adrenal hypoplasia and reduced LH and FSH levels in the pituitary (Ingraham et al., 1994; Shinoda et al., 1995). To assess directly its role in the pituitary, conditional deletion was performed in RP. This leads to a severe reduction in expression of LH and FSH. Reduction in gonadotrophins results expectedly in severely hypoplastic gonads and infertility (Zhao et al., 2001). Transcriptionally SF1 can directly bind the LH promoter to activate its expression and deletion of the SF1 binding site prevents expression (Keri,

Nilson et al., 1996). Co-operation with other factors is also important as synergy between SF1, PITX1 and Early Growth Response 1 (EGR-1), a zinc-finger TF, on the LH promoter is necessary to drive expression (Quirk et al., 2001). Additionally, FOXO1, a member of the FOXO family of Forkhead TF can directly binds SF1 to sequester it away from the LH promoter and therefore inhibits LH expression, possibly in response to metabolic cues (Arriola et al., 2012). Mutations in *SF1* are associated with 46XY sex reversal (Achermann et al., 2001) and ovarian insufficiency (Lourenço et al., 2009).

1.5.3 TPIT lineage

The T-Box 19 (Tbx19, also known as T-Box Factor Pituitary (Tpit)) lineage comprises two endocrine cell types, corticotrophs in AL and melanotrophs in IL (Figure 1.4). TPIT expression is first observed in RP at 12.5dpc just before differentiation of the first corticotrophs. The melanotroph lineage is not specified until TPIT becomes expressed in the dorsal region of RP at 15.5dpc (Japon et al., 1994; Lamolet et al., 2001). In addition to TPIT, both endocrine cell types also express PITX1 and the precursor of MSH and ACTH, POMC. Transcriptional regulation of *Pomc* in both cell types is controlled by TPIT that, along wit PITX1 binds to the CE3 element of the *Pomc* promoter (Lamolet et al., 2001). Neuronal differentiation 1 (NeuroD1), a basic Helix-Loop-Helix (bHLH) TF, is only and transiently expressed in corticotrophs where it can also act in tandem with PITX1 to induce the expression of POMC (Poulin et al., 2000; Poulin et al., 1997). In its absence, corticotrophs differentiation is slightly delayed (Lamolet et al., 2004). This is in contrast with the phenotype displayed by *Tpit* null mice, where there is a almost complete loss of POMC expression both in the anterior and intermediate lobes and, as a consequence, severe adrenal deficiency is observed, also seen in humans carrying *TPIT* mutations (Pulichino et al., 2003; Pulichino et al., 2003). NeuroD1 is still expressed in *Tpit* mutants, indicating that TPIT is required for terminal differentiation rather than cell commitment (Pulichino et al., 2003).

In addition to "promoting its own lineage", TPIT actively inhibits the emergence of gonadotrophs and rostral-tip thyrotrophs. *In vitro* TPIT and SF1 mutually repress each other transcriptional activities, by direct protein-protein interaction (Pulichino et al., 2003). In *Tpit* null IL, the relevance of this interaction is demonstrated by appearance of ectopic gonadotrophs and also rostral-tip thyrotrophs (Pulichino et al., 2003).

Recently, PAX7 has been shown to be responsible for the melanotroph specification of dorsal TPIT^{+ve} cells. Paired box 7 (PAX7) is a member of the PD TF family and is expressed in RP, in the future IL from 14.5dpc and maintained there as TPIT^{+ve} melanotrophs differentiate (Budry et al., 2012). Deletion of the gene results in a loss of melanotrophs, replaced by ectopic corticotrophs. This demonstrates its role as a selector of melanotroph fate, versus corticotroph cell identity (Budry et al., 2012). PAX7 acts through chromatin re-modeling, by allowing TPIT to bind on a subset of melanotroph specific binding sites (Budry et al., 2012). Interestingly PAX7 co-localises with SOX2 in a few cells in the post-natal pituitary, indicating a transitory SOX2^{+ve};PAX7^{+ve} state during melanotroph specification (Budry et al., 2012). In conclusion, TPIT is a marker of differentiation, promoting in particular the expression of genes that posttranslationally modify POMC into MSH or ACTH. Conversely PAX7 acts as a selector of melanotroph fate within the TPIT lineage.

By the end of gestation all endocrine cell type have differentiated in the pituitary. Post-natally, in rodents, the pituitary goes through a phase of rapid growth, allowing the gland to reach an adult size. During this phase progenitors and differentiated endocrine cells proliferate intensively.

1.6 Pituitary stem cell population

In order to meet the physiological demands of the body the pituitary must secrete hormones at varying levels. The pituitary has different ways of adapting its secretions; likely depending on how urgently, and how long for, the requirement needs to be met. The immediate need for hormones in physiological situations such as ovulation can be met via the release of intracellular stores of LH into the blood stream under GnRH hypothalamic control (Baird et al., 1976). Here, a tonic hypothalamic GnRH release into the hypophyseal portal system induces the LH pre-ovulatory surge. Another mechanism to increase hormone levels is the probably less immediate increase in hormone synthesis. This type of mechanism is likely to be important in longer periods of increased hormonal need such as the requirement for increased PrL during lactation (Escalada et al., 1996). Finally and likely in a longer term, the number of endocrine cells in the pituitary can be modulated. Newly generated cells can come from self-duplication of existing terminally differentiated endocrine cells, and this may be enough for normal cell turn-over (Langlais et al., 2013). In addition, new endocrine cells may come from differentiation of progenitors. In this section I will discuss the characterisation of the pituitary SC population, how it was isolated and the role it plays in the pituitary.

Chromophobes or hormone^{-ve} cells were first identified in the pituitary in 1969 (Yoshimura et al., 1969). Grafts of chromophobes cells in hypophysectomised rats induced appearance of acidophils (somatotrophs and lactotrophs) and basophils (corticotrophs, thyrotrophs and gonadotrophs) cells suggesting that they represented a 'reserve' cell type, able to differentiate (Yoshimura et al., 1969). In the literature, the putative pituitary SC population was proposed to reside in the marginal cell (MC) layer of the pituitary. In the AL, Folliculostellate cells (FS) were also proposed to have progenitor properties (Inoue et al., 1999; Shirasawa et al., 1983). Both populations, MC and FS express the glial fibrillary acid protein S100β. However these populations are extremely heterogeneous and were

subsequently shown to encompass the SC population in addition to other supporting cell types and undifferentiated endocrine cells (for review see Allaerts & Vankelecom., 2005).

1.6.1 *In vitro* characterisation of the pituitary SC population

1.6.1.1 Side population

The first study to identify a putative SC population in the pituitary was from Chen et al (2005). This group identified a 'side population' (SP) of cells in the AP characterized by their ability to exclude the dye Hoechst 3324. Significantly cells in SP did not express any hormones, and showed high levels of Stem cell antigen 1 (Sca1) (Figure 1.5), which is also expressed in SCs of the heart and mammary glands (Matsuura et al., 2004; Welm et al., 2002). Additionally, other SC markers such as *S100β, Oct-4, Nano*g and *Bim-1* are highly expressed in the Sca1 population, in contrast with the remaining population. Further characterization of the SP identified a $Sca1^{Hi}$ and non-$Sca1^{Hi}$ sub-populations (Chen et al., 2005, Chen et al., 2009). Self-renewal and differentiation potentials, two characteristics displayed *in vivo* by progenitors, can both be assessed *in vitro,* in spheres assays (Reynolds and Weiss., 1992). These assays have been used to identify progenitors in different tissues and organs (for review see Pastrana et al., 2011). Within the SP, the non-$Sca1^{Hi}$ population is the only one to display sphere-forming ability. These results suggest, along with marker analysis, that the non-$Sca1^{Hi}$ population contains progenitors (Chen et al., 2009), and overlaps with the progenitor population identified in Fauquier et al (2008) and Rizzoti et al (2013) (see below) (Figure 1.5).

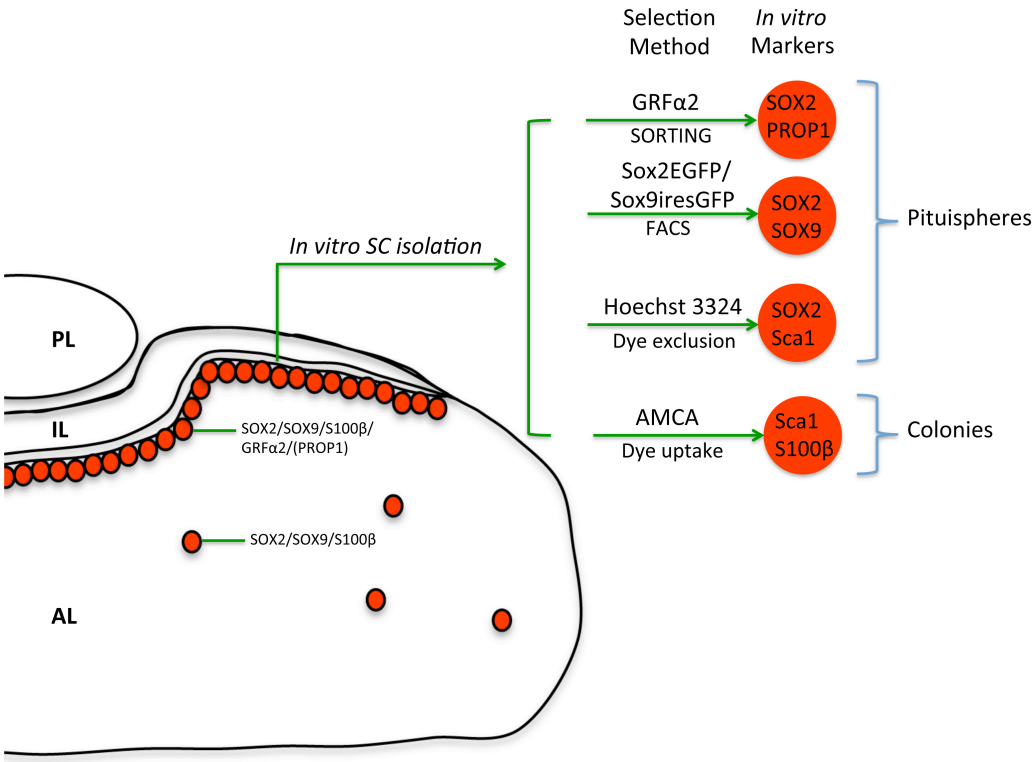


Figure 1.5: *In vitro* characterisation of proposed pituitary SC populations.

SOX2^{+ve} cells line the cleft of the adult pituitary and express SOX2, SOX9, GRFα2 and S100β. PROP1 is found in some of these cells only in the early postnatal period. SOX2 and SOX9^{+ve} cells are also found scattered in the parenchyma, where they can co-localise with S100β. Various selection methods have been used to isolate adult pituitary progenitors *in vitro*, resulting in different markers being used to identify these cells. Sphere and colony formation assays were used to show these cells to have progenitor characteristics.

1.6.1.2 SOX2;SOX9 population

A further step toward identification of the SC population in the pituitary came from Fauquier et al (2008). Progenitors were shown to express SOX2 and the SOXE protein SOX9, from embryonic stages to adulthood (Figure 1.5) (Fauquier et al., 2008). Moreover, pituispheres were also shown to express both proteins. Importantly, differentiation potential of cells within pituispheres was assessed in this study. By removing growth factors and allowing spheres to attach on Matrigel, this group was able to show that pituispheres could give rise to all AP endocrine cell types. Differentiation was preceded by upregulation of S100β, suggesting that FS cells may contain a transit amplifying population more defined towards a particular endocrine cell type (Fauquier et al., 2008). More recent studies have shown that

SOX2^{+ve} and SOX9^{+ve} cells are the only cells in the pituitary capable of forming pituisphere (Figure 1.5) (Rizzoti et al., 2013).

1.6.1.3 GPS population

Garcia-Lavandeira et al (2009), identified a sub-population of SOX2^{+ve} ;SOX9^{+ve} cells characterized by the co-expression of PROP1 and the Glial cell line-derived neurotropic factor (GDNF) receptor alpha 2 (GFRα2), that they named GPS (GFRα2/PROP/Stem) cells (Figure 1.5). These are found exclusively lining the cleft (MC layer). No fully differentiated endocrine cells express GPS markers, however 50% of the GFPs cells expressed the FS marker *S100β* (Garcia-Lavandeira et al., 2009). In the neonatal pituitary, proliferation is high, in particular in the GPS cells but it decreases quickly with age (Garcia-Lavandeira et al., 2009; Nolan et al., 1998). *In vitro*, the GFRα2^{+ve} population can form pituispheres, in contrast with the GFRα2^{-ve} population (Figure 1.5) (Garcia-Lavandeira et al., 2009). PROP1 is expressed in these cells and is also found in the non-Sca1^{Hi} population identified in Chen et al (2009), which also retained pituisphere-forming ability. Interestingly all PROP1^{+ve} cells express SOX2 and these are located around the lumen in neonatal mice, but not all SOX2^{+ve} cells express PROP1 (Figure 1.5). SOX2;PROP1 co-localisation decreases with age as PROP1 expression is quickly downregulated after birth (Yoshida et al., 2009). In conclusion the GPS population represents a small sub-population of SOX2^{+ve};SOX9^{+ve} progenitor population.

1.6.1.4 Pituitary colony forming cells

An alternative method to identify SC populations within a tissue is the ability to form adherent colonies *in vitro* (Figure 1.5). Dispersed adult AP cells can also form colonies and cells with this capacity represent 3.7% of the entire AP (Lepore et al., 2005). The pituitary colony forming cells (PCFC) also have a FS appearance and express S100β (Lepore et al., 2005), suggesting that they are part of the heterogeneous population of non-

endocrine FS cells discussed above. To demonstrate this, the fluorescent dipeptide β-Ala-Lys-Nε-AMCA was used as it is selectively taken up in FS cells (Otto et al., 1996). Selection of AMCA^{+ve} cells demonstrated that the colony forming ability was indeed restricted to this population (Figure 1.5) (Lepore et al., 2005). Further enrichment using fluorescence activated cell sorting (FACS) for Sca1^{+ve} cells indicated that 55% of the AMCA^{+ve} population expressed this marker (Figure 1.5) (Lepore et al., 2006). Expression of Sca1, S100β and AMCA suggested that these cells form part of the heterogeneous FS population, likely more defined towards a particular endocrine cell type. Further purification and transplantation of these cells into immuno-compromised mice resulted in the *in vivo* differentiation of somatotrophs, demonstrating the progenitor properties of this population (Lepore et al., 2007).

1.6.2 *In vivo* characterisation of the pituitary SC population

1.6.2.1 SOX2 and SOX9 lineage tracing

In vivo lineage tracing studies in different tissues have indicated that SOX2^{+ve} cells represent SC populations that give rise to terminally differentiated cell types (for review see Sarkar & Hochedlinger., 2013). Recent lineage tracing analyses have mostly relied on Cre recombinase and a ubiquitous reporter allele such as Rosa26Reporter (Srinivas et al., 2001). Upon Cre expression, typically under control of a SC or progenitor marker, a floxed STOP codon upstream of the reporter is recombined. The reporter is then expressed and will be maintained in the progeny of the mother cell. Inducible Cre recombinases allow an added level of control, as Cre expression can be switched on punctually, to assess progenitor properties at different times and in different situations (for review see Kretzschmar & Watt., 2012). This technique was used to follow the lineage of the SOX2^{+ve} cells in the embryonic and adult pituitary. Using an inducible system to label adult SOX2 cells in the pituitary with eYFP Rizzoti et al (2013) and Andoniadou et al (2013) revealed these cells self-renew and give rise to all

endocrine cell types in the pituitary. In addition to this SOX9^{+ve} cell lineage tracing using a similarly inducible system also resulted in eYFP^{+ve};Hormone^{+ve} cells in all endocrine cell types of the adult pituitary (Rizzoti et al., 2013). Previous studies suggested the adult SC population was distinct from the early progenitor population observed in RP (Gleiberman et al., 2008). Induction of Cre activity in SOX2^{+ve} and SOX9^{+ve} cells in the embryos and harvesting of the glands post-natally revealed that these cells not only give rise to all endocrine cell types but also to the SOX2^{+ve};SOX9^{+ve} SC population observed in the adult pituitary (Rizzoti et al., 2013). *In vivo* lineage tracing in these studies demonstrate that SOX2 and SOX9^{+ve} cells can both differentiate and self-renew and represent therefore a population of adult pituitary stem cells (Andoniadou et al., 2013; Rizzoti et al., 2013).

1.6.2.2 Regenerative potential of the adult stem cell lineage

Under normal conditions the adult pituitary has a very low turnover of cells, similar to the brain or heart at about 1.5% per day (Nolan et al., 1998). This is in contrast with the high mitotic activity observed in neonatal pituitaries reflecting a phase of rapid growth of the gland during the first weeks of life in rodents. Indeed the number of SOX2^{+ve} cells lining the lumen and the number of proliferative SOX2^{+ve} cells in this region are higher at birth, both progressively decreasing with age (Gremeaux et al., 2012). Moreover neonatal mice also have significantly more pituisphere forming cells; again this decreases with age (Gremeaux et al., 2012). Lineage tracing experiments also showed that progenitors mostly give rise to endocrine cells in the embryo and early post-natally. In adults SCs are mostly quiescent (Rizzoti et al., 2013; Andoniadou et al., 2013).

1.6.2.2.1 Organ ablation

Target organs of the pituitary send to the hypothalamus and pituitary negative feed-back cues in response to pituitary hormonal stimulation.

Target organ ablations suppress this negative feedback generally leading to a transient mitotic wave in the pituitary followed by an increase in corresponding pituitary hormone production. More precisely, adrenalectomies (Adx) and gonadectomies (Gdx) induce an increase in the mitotic index of the overall pituitary and a significant increase in the number of corticotrophs and possibly gonadotrophs (Ibrahim et al., 1986; Nolan & Levy., 2006). However the increase in the number of proliferating corticotrophs and gonadotrophs as a proportion of total dividing cells in Adx and Gdx mice is very small. In fact, proliferation is seen primarily in hormone^{-ve} cells response to organ ablation (Nolan & Levy., 2006). Moreover ablation of both adrenals and gonads does not have an additive proliferative effect (Nolan & Levy., 2006). This suggests that a population of undifferentiated progenitors proliferate and differentiate in response to target organ ablation (Nolan & Levy., 2006).

In agreement with these studies, SOX2^{+ve};SOX9^{+ve} were shown to proliferate in response to organ ablation studies (Rizzoti et al., 2013). Moreover, more pituispheres were obtained from animals that had undergone either Adx or Gdx, probably reflecting a stimulated, more proliferative SC population (Rizzoti et al., 2013). Importantly lineage tracing in *Sox9CreER^{T2};R26R^{eYFP}* mice following Adx demonstrated that, in addition to proliferation, progenitors also differentiate and give rise to a proportion of the newly generated corticotrophs (Rizzoti et al., 2013). This indicates that the SOX2^{+ve};SOX9^{+ve} population have a regenerative potential that can be stimulated by physiological demand.

1.6.2.2.2 Cellular ablation

An alternative method of demonstrating the regenerative capacity of the pituitary and requirement of the SC population is cellular ablation. Somatotroph ablation using GHCre/iDTR mice, in which the diphtheria toxin receptor is specifically expressed in GH^{+ve} cells, results in a large increase in the size of the SP (Fu et al., 2012). In addition, both the number of SOX2^{+ve}

cells in the MC layer, and their mitotic index are increased (Fu et al., 2012). Another report from the same laboratory used the same technique to ablate lactotrophs in the adult pituitary (Fu & Venkelecom., 2012). Similarly the number and proliferative capacity of SOX2 cells in the MC layer increased. Limited regeneration of ablated endocrine cells was observed in both studies, new cells were suggested, but not demonstrated, to originate from the SOX2 SC population.

An elegant ablation study used *invloxP;POMC-Cre* mice. In these mice Cre expression triggers a chromosomal re-arrangement inducing apoptosis, only if the cells divide (Langlais et al., 2013). POMC is expressed in differentiated corticotrophs and *invloxP;POMC-Cre* mice display a significant decrease in this cell population. This demonstrates that corticotroph cell turn-over occurs via self-duplication of terminally differentiated cells (Langlais et al., 2013). Significantly the mitotic index of $SOX2^{+ve}$ cells is not increased in these mice, suggesting that SC are not stimulated by the gradual loss of a proportion of corticotrophs over a long period. In contrast, following Adx in *invloxP;POMC-Cre* mice, new $TPIT^{+ve}$ cells are produced. Generation of new cells in this model shows that these do not originate from pre-existing $POMC^{+ve}$ corticotrophs. Moreover, this renewal is associated with an increase in the mitotic index of $SOX2^{+ve}$ cells. These data suggest that the regenerative potential of the SC population is stimulated, and that this only happens when an acute challenge is induced (Langlais et al., 2013), in agreement with lineage tracing experiments (Rizzoti et al, 2013).

In conclusion the $SOX2^{+ve}$;$SOX9^{+ve}$ population in the adult pituitary comprises a SC population and also more committed progenitors. The role of SOX2 in these adult SCs and more specifically embryonic progenitors is unknown.

1.7 Thesis Outline

During pituitary development SOX2 is initially expressed in early progenitors. Initially highly proliferative, RP progenitors later exit cell cycle and give rise to all the endocrine cells of the pituitary. As development progresses and cells differentiate, SOX2 is downregulated, however a population of $SOX2^{+ve}$ cells remains, lining the pituitary cleft. In the adult gland these cells represent a SC population, capable of self-renewal and differentiation. In humans and mice mutations in *SOX2/Sox2* are associated with hypopituitarism. Consequently we decided to examine the role of SOX2 in pituitary development.

The work presented in this thesis aims to investigate the role of SOX2 in pituitary development. As deletion of the gene is early embryonic lethal, we employed the Cre LoxP system to conditionally delete a floxed allele of *Sox2*. Disruption of SOX2 expression in the overlying VD has been shown to affect RP development. As a consequence we identified appropriate Cre drivers that were not active in the VD. The first results chapter (Chapter 3) outlines the analysis of two different Cre drivers and their respective advantages and disadvantages as tools to delete *Sox2* in RP. Subsequently in Chapter 4 we perform analysis of the phenotypes resulting from *Sox2* deletion using both Cre drivers. These include characterisation of morphological defects in early and late stages of RP development, identification of factors involved during early RP development and affected by loss of SOX2, and examination of endocrine cell differentiation. Finally in chapter 5 we focus on a specific defect caused by *Sox2* deletion, in the IL. In this chapter we aim to investigate further the mechanisms underlying this phenotype. To achieve this, we perform a genetic interaction assay using *p27kip1* null mutants to rescue the IL phenotype.

The studies presented in this thesis investigate the role of SOX2 in pituitary development by analysing the consequences of its deletion in RP. In the final

chapter (chapter 6) we propose a model for its role in the developing gland and discuss further experiments.

2. Materials and Methods

2.1 Transgenic Mouse Lines

2.1.1 Animal stocks and husbandry

Mice were bred onsite at the NIMR in E type cages in the Laidlaw Green animal unit. All mice stocks and scheduled procedures were carried out in accordance with Home Office regulations. Mice were kept in a 12h light/dark cycle and data collected from virgin male and female mice unless otherwise stated.

2.1.2 Genetically modified mouse strains

Genetic alteration of gene expression within specific cell types was achieved using the bacteriophage P1 Cre recombinase/LoxP system. The animals used in this study are established transgenic mouse lines that have been previously described (Table 2.1).

2.1.3 Genotyping and PCR amplification

Genotyping was performed by polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) extracted from ear punches taken from mice at three weeks of age. Appropriate primers were used for amplification of each allele (Table 2.2). Ear punches (adult) or tail tips (embryo) were digested in 20 micro liters (μl) of DNAreleasy mix (Anachem) incubated at 75 degrees centigrade (°c) for 5 minutes, followed by enzyme inactivation by incubating at 95°c for 2 minutes. The DNA was then amplified using Thermoprime *Taq* DNA polymerase (Thermo Scientific) and added to a master mix with the following reagents: 0.5μl genomic template DNA, 0.2 U/μl *Taq* polymerase, 1x reaction buffer, 0.2 millimolar (mM) deoxynucleotide triphosphates (dNTPs), 1.5mM $MgCl_2$, 0.6mM of each primer and PCR-grade water up to a reaction volume of 25μl.

The thermocycle consisted of 35 cycles of 95°c for 30s, 58°c for 30s and 72°c for 1 minute per kilobase of amplification. PCR products were then visualised and size verified on a 1.5% w/v agarose/SB (Brody & Kern, 2004), Tris-acetate-EDTA (TAE) (1xTAE solution: 40mM Tris-acetate, 20mM acetic acid, 1mM EDTA in deionized water) gel with 5ng/ml ethidium bromide. See table 2.2 for genotyping primers and corresponding PCR product sizes.

Name of Allele	***Description of Genetic Manipulation***	***Genetic Background***	***Source and Reference***
FoxG1^{Cre}	Knock-in of Cre recombinase into the FoxG1 locus, replacing one copy of *FoxG1*	129SvJ	(Hébert & McConnell., 2000a)
Nkx3.1^{Cre}	Knock-in of Cre recombinase into the *Nkx3.1* locus, replacing one copy of *Nkx3.1*	C57BL/6	Gift from M. Shen (Lin et al., 2007)
p27$^{-/-}$	Targeted disruption of Exons 1 and 2 of *p27*, replacing these with a pgk-neo cassette	C57BL/6	(Fero et al., 1996)
Rosa26^{eYFP}	*LoxP* sites flanking a STOP cassette preceding enhanced yellow fluorescent protein (YFP) inserted into the *Rosa26* locus	C57BL/6	(Srinivas et al., 2001)
Sox2^{COND}	*LoxP* sites flanking the *Sox2* open reading frame	C57BL/6	(Taranova et al., 2006)
GBS^{GFP}	8GBS-*hsp68*-eGFP transgene inserted into the BamH1 site of pJC13-1 locus	C57Bl/6	(Balaskas et al., 2012)

Table 2.1: Mouse strains.

Primer Set	*Sequence*	*Genotypes for*	*Product Size (bp)*
Cre-Frd *Cre*-Rev	CCAGCTAAACATGCTTCATC CGCTCGACCAGTTTAGTTAC	*Cre* allele	300
p27-WT-Frd *p27*-WT-Rev	TGTCAAACGTGAGAGTGTCTAACGG AACCCAGCCTGATTGTCTGACGAG	WT *p27* allele	400
p27-KO-Frd *p27*-KO-Rev	CCTTCTATCGCCTTCTTGACG TGGAACCCTTGTGCCATCTCTAT	KO *p27*	550
Sox2-Frd *Sox2*-Rev	GCTCTGTTATTGGAATCAGGCTGC CTGCTCAGGGAAGGAGGGG	*Sox2* WT	382
Sox2-flox-Frd *Sox2*-flox-Rev	CAGCAGCCTCTGTTCCACATACAC CAACGCATTTCAGTTCCCCG	Floxed *Sox2* allele	297
5'-*Rosa26* 3'-*Rosa26*-1 3'-*Rosa26*-2	AAAGTCGCTCTGAGTTGTTAT GCGAAGAGTTTGTCCTCAACC GGAGCGGGAGAAATGGATATG	WT and transgenic *Rosa* allele	300 525

Table 2.2: PCR primers.

2.2 *Ex vivo* analysis

2.2.1 Immunofluorescence

Between 10.5 and 18.5dpc, embryos were fixed in 4% paraformaldehyde (PFA) (Sigma) overnight at 4°C. 14.5dpc embryos had a hole opened at the top of the skull and 16.5-18.5dpc embryos has their skulls opened and brains removed to allow full penetration of PFA and complete fixation of the developing pituitary. Tissues were cryopreserved in sucrose (20%) overnight at 4°C, embedded in OCT mounting medium (VWR Chemicals) and stored at -80°C. Between 10.5dpc and 14.5dpc embryos were embedded and sectioned in a sagittal orientation (see figure 1.3 for a schematic representation). 16.5dpc and 18.5dpc pituitaries that had been removed from the skull or exposed to the fixative by removal of the brain but left in situ were embedded and sectioned in a coronal orientation (see figures 1.3 and 1.4 for schematic representation). All embryos/pituitaries were sectioned serially in 12 micrometre (μm) sections.

12μm sections were blocked at room temperature in blocking buffer (1x phosphate buffered saline (PBS), 0.1% triton x-1000, 10% fetal bovine serum (FBS)) (BB) for one hour. Subsequently primary antibodies were added at an appropriate dilution (Table 2.3). Primary antibodies were added individually, except when all hormones were to be identified on the same section. In this case a mix of all five hormonal antibodies (rbPOMC, rbTSH, rbGH, rbLH, rbPrL) were added together (Figure 4.3). Primary antibodies were incubated overnight at 4°C. The primary antibody solution was then washed off in three changes of PBT (1xPBS, 0.1% triton x-1000) for five minutes each. The appropriate secondary antibody was then added (Table 2.4), along with 4',6-diamidino-2-phenylindole (DAPI) (1:1000), and incubated at room temperature for one hour. Finally the secondary antibody was removed and the slides washed with three changes of PBS for five minutes each and cover slip mounted using Aqua-poly/Mount (Polysciences, Inc.).

Tissues that required antigen retrieval were fixed in 4% PFA overnight and embedded in paraffin. Sections were de-waxed in two 10-minute washes in histoclear (National Diagnostics) and rehydrated by successive 5-minute incubations in decreasing ethanol solutions (100%-99%-95%-70%-50%). The slides were then incubated for 20 minutes in 1x target retrieval buffer (Dako) at 120°C in a decloaker. Following this, the slides were processed following the protocol described above. To assess cell proliferation, Bromodeoxyuridine (BrdU) (Sigma) or ethynyl-2-deoxyuridine (EdU) (Molecular Probes) was injected in pregnant females at an appropriate concentration (see below). Embryos were harvested one hour after injection. BrdU immunostaining was carried out on 12μm sections after hydrolysis at 37°C in 2 molar (M) hydrochloric acid (HCL) (Sigma) for one hour then neutralisation with borate buffer (Boric acid, 100mM (BDH Chmicals), NaCl (sodium chloride) 75mM (Fisher Scientific), Borax 25mM (Sigma)). The same immunohistochemical protocol was performed as described above, using rat anti-BrdU (Table 2.3). EdU assays were performed using the 'Click-iT EdU Alexa Fluro 594 Imaging kit' (Invitrogen).

TUNEL assays were performed using the ‘Apop Tag Fluorescein in Situ Apoptosis Detection Kit’ (Millipore).

Primary Antibody Antigen	*Host Species*	*Final Dilution*	*Source*
BrdU	Rat	1:100	Abcam
GFP	Rat	1:500	Tesque Nacalai
GFP	Goat	1:1000	Abcam
GFP	Rabbit	1:1000	Abcam
GH	Rabbit	1:100	National Hormone and Peptide Program
GR	Rabbit	1:100	Santa Cruz
ISL1	Mouse	1:300	Gift (J. Briscoe)
LHβ	Rabbit	1:500	NHPP
LHX3		1:500	Abcam
P27kip1	Mouse	1:30	BD Transduction Laboratories
PAX7	Mouse	1:50	DSHB
POMC	Rabbit	1:500	NHPP
POMC	Mouse	1:000	Fitzgerald Industries
POU1F1	Goat	1:500	Gift (Simon Rhodes)
PRL	Rabbit	1:200	NHPP
SF1		1:300	Cell Signaling Solutions
SIX3	Rabbit	1:500	Rockland
SIX6	Rabbit	1:500	Sigma
SOX2	Goat	1:500	Immune Systems Ltd.
SOX9	Goat	1:200	R&D
TBX19	Rabbit	1:1000	Gift (D. Drouin)
TSHβ	Rabbit	1:500	NHPP

Table 2.3: Primary antibodies.

Secondary Antibody	*Fluorophore*	*Final Dilution*	*Source*
Donkey anti-goat	Alexa-555/488/647	1:1000	Molecular Probes
Donkey anti-rabbit	Alexa-555/488/647	1:1000	Molecular Probes
Donkey anti-mouse	Alexa-488	1:1000	Molecular Probes
Donkey anti-rat	Alexa-488	1:1000	Molecular Probes
Goat anti-mouse	Alexa-488	1:1000	Molecular Probes
Goat anti-guinea pit	Alexa-555/488	1:1000	Molecular Probes

Table 2.4: Secondary antibodies

2.3 *In vivo* analysis

2.3.1 BrdU and EdU injection

BrdU (10mg/ml) was injected into the peritoneum of pregnant mice. Following a 1h incubation period the mice were culled by SK1 (schedule 1 kill) and embryos processed. EdU (10mg/ml) was injected into the peritoneum of pregnant mice. Following a 1h incubation period the mice were culled by SK1 and embryos processed.

2.3.2 Harvesting and preparation of embryonic and adult tissues

All mice were culled by SK1. Embryos were harvested by cesarean section and culled by SK1 from 14.5dpc onward. 9.5dpc to 12.5dpc embryos were fixed whole in 4% PFA, 14.5 to 18.5dpc embryos had the head removed and fixed in 4%PFA.

2.4 Image processing and analysis

Images were taken using a Leica SPE confocal microscope using 10X and 20X objective lenses. Each fluorescent tag was imaged separately using the 405, 488, 561 and 649 channels respectively. Confocal images were taken as Z-stacks and processed using Fiji (Schindelin et al., 2012) and Adobe Photoshop CS4.

2.5 Statistical analysis

The number of $BrdU^{+ve}$ nuclei in RP at 12.5dpc was quantified in three embryos. Each embryo was sectioned sagittally and the number of $BrdU^{+ve}$ nuclei was counted on three sections, one mid-line and two lateral RP sections. Quantification of the number of $BrdU^{+ve}$ nuclei in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos was restricted to the dorsal part of RP, in the region where $Nkx3.1^{Cre}$ is active. Quantification of the number of $BrdU^{+ve}$ nuclei in

Sox2$^{fl/fl}$*;FoxG1*$^{Cre/+}$*;R26R*$^{eYFP/+}$ embryos was not restricted due to the ubiquitous activity of *FoxG1*^{Cre} in RP. The number of BrdU^{+ve} nuclei is presented as a percentage of the total number of DAPI^{+ve} nuclei in the restricted region of *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos, or whole RP in *Sox2*$^{fl/fl}$*;FoxG1*$^{Cre/+}$*;R26R*$^{eYFP/+}$ embryos, with error bars representing the standard error of the mean. The average number of DAPI^{+ve} nuclei counted per *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ embryo was 482, reducing to 322 in *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos. In *Sox2*$^{fl/+}$*;FoxG1*$^{Cre/+}$*;R26R*$^{eYFP/+}$ embryos the average number of DAPI^{+ve} nuclei counted was 1140 per embryo. This reduced to 718 in *Sox2*$^{fl/fl}$*;FoxG1*$^{Cre/+}$*;R26R*$^{eYFP/+}$ embryos.

EdU^{+ve} nuclei quantification at 18.5dpc was restricted to the IL as defined by its localisation and structure. The IL is easily identified between the AL and PL due to its separation from the anterior pituitary via the lumen, and distinguishable form the posterior pituitary as cell morphology is very different. This is true both in control and mutant pituitaries. The number of EdU^{+ve} nuclei was counted on three sections per pituitary, in three pituitaries per genotype. The number of EdU^{+ve} nuclei is represented as a percentage of the total number of DAPI^{+ve} nuclei, with error bars representing the standard error of the mean. The average number of DAPI^{+ve} nuclei counted per embryo within each genotype was; WT: 578, *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$: 375, *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$: 793 and *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$: 540.

Quantification of the total number of DAPI^{+ve} nuclei in the IL was performed on 2/3 pituitaries per genotype. Each pituitary was entirely serially sectioned and the number of DAPI^{+ve} nuclei counted on three equivalent sections. Data was presented as the total number of DAPI^{+ve} nuclei for each genotype; WT, *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$, *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$.

Quantification of the percentage of POMC^{+ve} cells in the IL of WT, *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$, *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos was performed on three pituitaries per genotype. Each pituitary was entirely serially sectioned

and the number of each endocrine cell type counted on three equivalent sections. Number of POMC^{+ve} cells was presented as a percentage of the total amount of DAPI^{+ve} nuclei, with error bars representing the standard error of the mean. The average number of DAPI^{+ve} nuclei counted per embryo in each genotype was; WT 578, *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* 375 and *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* 793.

Quantification of the percentage of each endocrine cell type within the AL and IL was performed on three pituitaries per genotype. Each pituitary was entirely serially sectioned and the number of each endocrine cell type counted on three equivalent sections. Each endocrine cell type was presented as a percentage of the total amount of DAPI^{+ve} nuclei within the lobe in which the population is present, with error bars representing the standard error of the mean. The IL was defined by its localization and structure (see above) and melanotrophs counted as a percentage of total number of DAPI^{+ve} nuclei in the IL only. The average number of DAPI^{+ve} nuclei counted per embryo in the AL was 2674. In the IL the average number of DAPI^{+ve} nuclei counted per embryo was 578.

The number of SOX2^{+ve};eYFP^{+ve} cells in the RP of *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos was quantified as a percentage of the total number of SOX2^{+ve} nuclei at 12.5dpc and 18.5dpc. In *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos this was quantified in three embryos. Each embryo was sectioned sagittally and the number of SOX2^{+ve};eYFP^{+ve} cells was counted on three sections, one mid-line and two lateral RP sections. In *FoxG1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos quantification was only performed on one embryo due to the ubiquitous activity profile of the Cre driver. This embryo was sectioned sagittally and the number of SOX2^{+ve};eYFP^{+ve} cells was counted on three sections, one mid-line and two lateral RP sections. The number of SOX2^{+ve};eYFP^{+ve} cells is represented as a percentage of the total number of SOX2^{+ve} nuclei, with error bars representing the standard error of the mean. The average number of SOX2^{+ve} nuclei counted per *Nkx3.1^{Cre};R26R$^{eYFP/+}$* embryo was 75 at 12.5dpc

and 290 at 18.5dpc. The total number of SOX2^{+ve} nuclei counted in the *FoxG1^{Cre};R26R$^{eYFP/+}$* embryo was 102, at 10.5dpc.

The number of hormone^{+ve};eYFP^{+ve} cells for each endocrine cell type in *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos was quantified as a percentage of the total number of each endocrine cell type at 18.5dpc. Quantification was performed on three pituitaries except for each endocrine cell type, except for somatotrophs, which were only counted on two pituitaries. Each embryo was sectioned sagittally and the number of hormone^{+ve};eYFP^{+ve} cells was counted on three sections, one mid-line and two lateral RP sections. The number of hormone^{+ve};eYFP^{+ve} cells is represented as a percentage of the total number of each endocrine cell population, with error bars representing the standard error of the mean. The average number of endocrine cells counted per cell type in *Nkx3.1^{Cre};R26R$^{eYFP/+}$* embryo was; gonadotrophs 37.6, lactotrophs 19.3, thyrotrophs 74, corticotrophs 102.3, somatotrophs 283 and melanotrophs 178.

All statistical analyses were done using the Graph Pad Prism software. Unpaired Student t-tests were used to compare two groups of data and significance was represented according to the p-values. 0.05 = *, 0.01 = **. 0.001 = ***.

3. *Nkx3.1^{Cre}* and *FoxG1^{Cre}* Activity Profile in Rathke's Pouch

3.1 Introduction

Accurate deletion of *Sox2* in RP is essential for a correct assessment to be made as to its role in RP progenitors. Various considerations have to be taken into account when selecting the correct tool to achieve this. These include the timing of deletion, as SOX2 is globally expressed in RP from its induction up until 11.5dpc, thereafter it becomes restricted dorsally. Deletion in this time period will provide a better understanding as to its role in the true RP progenitors, that give rise to all endocrine cell types (Rizzoti et al., 2013). Secondly spatial considerations have to be taken into account due to the wide expression of SOX2 in other tissues. A broad field of deletion may affect global embryonic development and thus the embryo may die before RP induction, or RP formation may be directly affected by *Sox2* deletion in surrounding tissues.

An example of the need for correct temporal and spatial deletion is the lethality that occurs in *Sox2* homozygous null mutant embryos. This is observed at peri-implantation stages, as the protein is cell-autonomously required for both the epiblast, from which the embryo derives, and in extraembryonic tissues (Avilion et al., 2003). An earlier role in the ICM may be masked by the presence of maternal cytoplasmic SOX2 protein, imported into the nucleus of zygotes at the two-cell stage (Avilion et al., 2003). Embryonic lethality of *Sox2* null embryos prevented the study of later roles of SOX2; therefore alternative methods have been used to look at the role of SOX2 in different contexts.

As development proceeds SOX2 becomes largely restricted to the early CNS and by 9.5dpc is expressed throughout the brain, neural tube and sensory placodes along with other *SoxB1* genes (Avilion et al., 2003, Wood & Episkopou., 1999). Significantly, *Sox2* is expressed in the VD and subsequently in the infundibulum upon its formation (Zhao et al., 2012). The

importance of the VD in RP development cannot be understated. Expression and secretion of a number of morphogens, including BMF4, FGF8/10, Wnt5A and SHH from the VD are essential for the correct development of RP (Mathias Treier et al., 1998 Ericson et al., 1998, Potok et al 2008, Wang et al., 2010). Homozygous deletion of *Sox3* on a *Sox2* heterozygous background prevents hypothalamus formation, in addition to shifting FGF10 expression anteriorly and completely down regulating SHH. Moreover RP formation is severely impaired as a consequence (Zhao et al., 2012). Embryos heterozygous for a null mutation in *Sox3* alone display pituitary hypoplasia, extra clefts and altered FGF8 and BMP4 expression (Rizzoti et al., 2004). Similarly, *Sox2* heterozygous mice have a bifurcated RP and clefts in the adult pituitary in addition to being hypoplastic (Kelberman et al., 2006b). The similarity in phenotype between *Sox2* and *Sox3* heterozygous mice suggest the defects are a result of VD abnormalities instead of primary pituitary defects, as SOX3 is not expressed in RP. Thus wild-type levels of SOX2 expression in the VD and infundibulum are essential in promoting correct morphogen secretion and as a result correct RP development, whereas it would appear that heterozygous levels of SOX2 are at least mostly sufficient for RP development. Consequently, to determine the precise role of SOX2 in RP progenitors requires having a conditional mutation restricted to these cells, as loss of even one allele in the overlying VD would result in a phenotype not solely derived from loss of SOX2 in RP.

To achieve the correct temporal and spatial deletion of *Sox2,* advantage was taken of the conditional method of gene deletion using LoxP flanked *Sox2* alleles (Taranova et al., 2006), which can be excised by Cre recombinase. In this system the Cre coding region is placed downstream of the promoter of a gene chosen because of its specific pattern of expression, such that the Cre recombinase will be expressed with the same pattern. This “Cre-driver” construct can be derived by gene targeting, placing it into the endogenous locus, alternatively it can be constructed *in vitro* and randomly inserted into the genome as a transgene. Deletion of *Sox2* has been achieved this way in a number of cell types including the eye (Smith et al., 2009), teeth (Juuri et al.,

2013), glia (Gomezmez-Lopez et al., 2011) and germ cells (Campolo et al., 2013). Several Cre drivers exist with active recombination in RP. These include *Pit1-Cre* (Gaston-massuet et al., 2011), *POMC-Cre* (Balthasar et al., 2004), *PrL-Cre* (Castrique et al., 2010) and *GH-Cre* (Nasonkin et al., 2010). The spatial restriction of these Cre drivers to RP provides an extremely accurate way of deleting conditional alleles in these lineages. Nevertheless these Cre drivers would be unable to delete *Sox2* in early RP progenitors due to their late onset of activity, which occurs exclusively in terminally differentiating or differentiated cells.

The NK3 homeobox 1 (NKX3.1) protein is a mammalian transcription factor homologous to Bagpipe in Drosophila. NKX3.1 is expressed in multiple tissues in mice including somites, blood vessels, midgut and the male urogenital tract (Bieberich et al., 1996; Sciavolino et al., 1997; Tanaka et al., 1999) . First expressed at 9.5dpc in RP and maintained until 14.5dpc, it is present in a subset of dorsally restricted progenitors, where its function is unknown (Kioussi et al., 1999; Mathias Treier et al., 1998). The *Nkx3.1-LacZ* targeted allele is active in all tissues described above in addition to RP where, like endogenous NKX3.1 expression, activity is first observed at 9.5dpc. Thereafter activity becomes restricted to the dorsal region of RP at 16.5dpc (Schneider et al., 2000). Significantly *Nkx3.1-LacZ* is not observed in the VD or infundibulum (Schneider et al., 2000). A similarly targeted allele driving Cre recombinase has also been generated, providing the possibility to conditionally delete genes in the areas in which LacZ reporter expression was observed (Lin et al., 2007). *Nkx3.1*^{Cre} fills both requirements to successfully delete *Sox2* in RP. Firstly, unlike the Cre drivers listed above it fits the temporal requirement, as it will be active in the early RP progenitors (Treier et al., 1998; Schneider et al., 2000). Secondly and most importantly it fits the spatial requirement of not being active in the overlying VD; thus any phenotype associated with *Sox2* deletion in this area will be avoided.

The spatially restricted pattern of *Nkx3.1*^{Cre} activity in the dorsal region of RP prevents global deletion of *Sox2* during the early stages of pituitary

devolvement. Forkhead box G1 (FOXG1) is a TF that is expressed primarily in the telencephalon (Tao & Lai., 1992). It is first expressed at E8.0 in the anterior neural ridge that gives rise in particular to the hypophyseal placode (Shimamur et al., 1995). Hébert & McConnell (2000) produced a targeted *FoxG1*^{Cre} mouse line, in which the ORF of FoxG1 was replaced by the Cre recombinase. *FoxG1*^{Cre} mice bred with a R26R$^{eYFP/eYFP}$ line revealed that its pattern of activity matched closely the endogenous pattern of FOXG1 expression, providing the mice were bred on a 129/SvJ or Swiss Webster background to avoid ectopic activation, which has been reported to occur on other backgrounds (Hébert & McConnell., 2000). Due to its activation pattern *FoxG1*^{Cre} has primarily been used to delete genes in the forebrain, in particular *Sox2*, resulting in forebrain defects including loss of NKX2.1 and SHH expression (Ferri et al., 2013). More detailed lineage tracing was carried out by Wang et al (2010), showing LacZ reporter expression throughout the invaginating RP at 9.5dpc. This was maintained in RP at 12.5dpc showing global *FoxG1*^{Cre} activity in RP, however at this stage there are a few LacZ^{+ve} cells scattered in the infundibulum (Wang et al., 2010). The benefit of using *FoxG1*^{Cre} to delete *Sox2* in RP is the extremely early and ubiquitous pattern of activity. This will allow for global deletion of *Sox2* in all RP progenitors before the pouch forms at 11.0dpc and before the beginning of endocrine cell differentiation at 11.5dpc - 13.5dpc (Davis et al., 2011). Nevertheless the mild ectopic activity in the VD may disrupt ligand secretion in this region. Using both *Nkx3.1*^{Cre} and *FoxG1*^{Cre} will allow us delete *Sox2* with greater spatial and temporal specificity.

3.2 Results

3.2.1 *Nkx3.1^{Cre}* activity profile

In order to verify the activity profile of *Nkx3.1^{Cre}* and determine its usefulness as a means to study SOX2 function in RP, it was first crossed onto a Rosa26 Reporter eYFP (R26ReYFP) strain (Srinivas et al., 2001). eYFP was first detected at 10.5dpc in RP, but in only a small number of cells (Figure 3.1Ai), suggesting that any phenotype resulting from deletion of *Sox2* is likely to be mild and indistinguishable from WT embryos (Figure 3.1). In addition to this, eYFP was found to be expressed in the mesenchyme surrounding RP at 10.5dpc (Figure 3.1Ai). This mesenchyme is a source of BMP2, which is known to directly influence RP development (Ericson et al., 1998, see chapter 1). However, as SOX2 is not expressed in these cells, its loss will not have any consequences on RP development. By 12.5dpc the activity profile of *Nkx3.1^{Cre}* has greatly increased and eYFP can be seen in cells primarily in the dorsal and ventral regions of RP, overlapping with but not encompassing the entire SOX2^{+ve} population (Figure 3.1 Biii). Indeed, at this stage, 69.6% (±6.5, n = 3) of SOX2^{+ve} cells are YFP^{+ve} (Figure 3.2). By 18.5dpc the number of SOX2^{+ve}/YFP^{+ve} cells is very similar to that seen at 12.5dpc, at 61.1% (±6.0, n = 3) (Figure 3.1Biii, Eiii). Previous data indicated that NKX3.1 is expressed at least until 14.5dpc in the dorsal region of RP (Mathias Treier et al., 1998). This suggests that the *Nkx3.1^{Cre}* will reach its maximal activity between 12.5-14.5dpc. Moreover, as activity of *Nkx3.1^{Cre}* only becomes significant at 12.5dpc, the role of SOX2 in RP at earlier stages will not be revealed. Importantly, no eYFP^{+ve} cells were observed in the VD directly overlying RP at any stage examined, however a handful of eYFP^{+ve} cells were observed in the PL of 18.5dpc embryos. Consequently any phenotype seen in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos will be a result of specific and exclusive deletion of *Sox2* in RP.

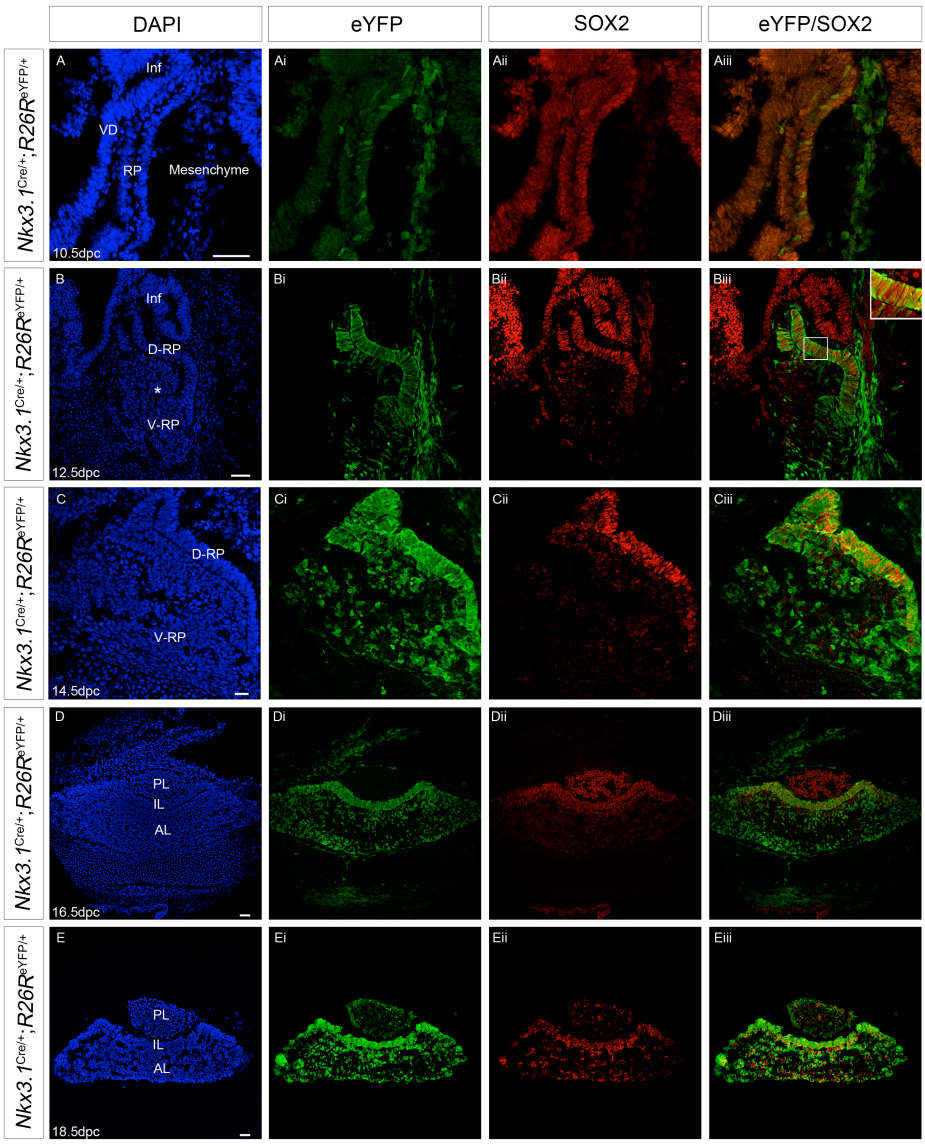


Figure 3.1: *Nkx3.1^{Cre}* activity profile.

Immunofluorescence stain for eYFP (green), SOX2 (red) counterstained with DAPI (blue) between 10.5-18.5dpc. *Nkx3.1$^{Cre/+}$* males were bred with *R26R$^{eYFP/eYFP}$* females to produce *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos. eYFP can be seen from 10.5dpc (Ai) and increases until 12.5dpc (Bi). SOX2 is ubiquitously expressed in RP from 10.5dpc but becomes dorsally restricted from 12.5dpc (Aii and Bii). By 16.5dpc and 18.5dpc SOX2 is found in the future IL and scattered in the parenchyma (Dii and Eii). The main area of eYFP expression is in the dorsal side of RP at 12.5dpc (Bi). eYFP co-localises with SOX2^{+ve} progenitors (inset, Biii) increasingly until 12.5dpc and thereafter it co-localises with SOX2 in the dorsal region of RP in the future IL, however at all stages not all SOX2^{+ve} cells are eYFP^{+ve} (Aiii – Eiii). Sections at 10.5dpc, 12.5dpc and 14.5dpc are sagittal. Sections at 16.5dpc and 18.5dpc are coronal (see figure 1.3 and 1.4 for schematic representation). VD = Ventral Diencephalon, Inf = Infundibulum, RP = Rathke's Pouch, PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. D-RP = Dorsal Rathke's Pouch and future IL, star = definitive RP and V-RP = Ventral Rathke's Pouch. Scale = 50μm.

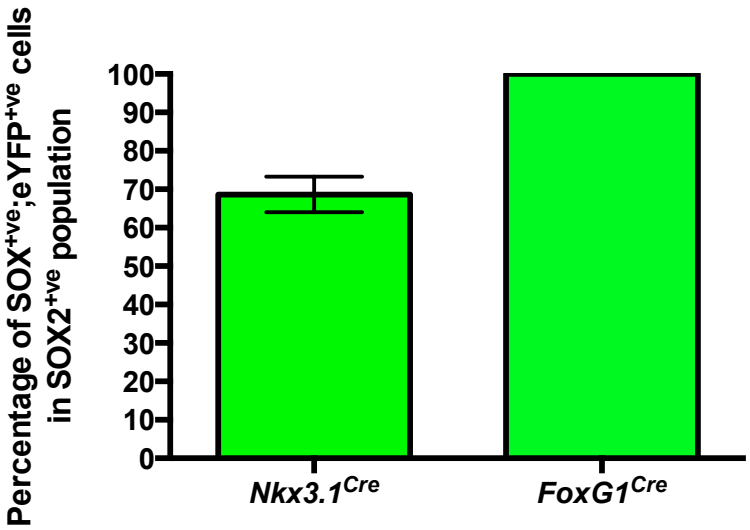


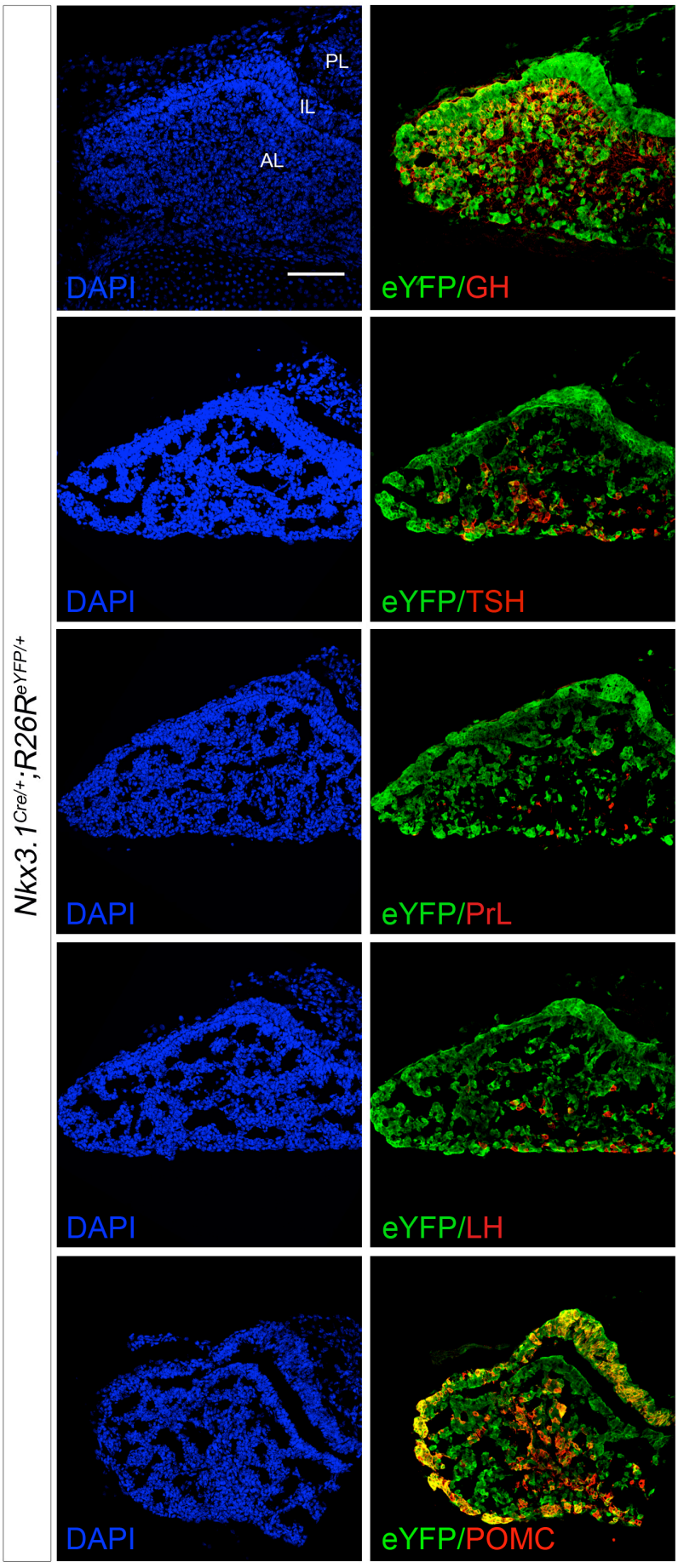
Figure 3.2: Percentage of SOX2^{+ve} cells that express eYFP with either Cre driver

68.3% (±6.5, n = 3) of SOX2^{+ve} cells co-express eYFP at 12.5dpc in *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos. 100% (n = 1) of SOX2^{+ve} cells co-express eYFP at 10.5dpc in *FoxG1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos.

3.2.2 *Nkx3.1^{Cre}* activity profile in terminally differentiated endocrine cell populations

Examination of *Nkx3.1^{Cre}* early activity profile indicated that only a subset of differentiated endocrine cells would be in the progeny of NKX3.1^{+ve} cells (Figure 3.1Eiii, 3.3A). We would expect a subset of each endocrine lineage to be affected, as progenitors for all endocrine lineages leave the progenitor region roughly at the same time, to differentiate and colonize the developing anterior lobe (Davis., et al 2011). We therefore decided to determine the proportion of cells originating from NKX3.1^{+ve} progenitors within each endocrine lineage by quantifying the percentage of each endocrine cell type that was eYFP^{+ve} in *Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* 18.5dpc embryos. The dorsal region of the early RP, where the highest levels of *Nkx3.1^{Cre}* activity are present at 12.5dpc, will give rise to the IL (Figure 3.1Ai). Accordingly, the number of POMC^{+ve};eYFP^{+ve} cells (melanotroph precursors) in the IL is high, 92.0% (±1.8, n = 3) by 18.5dpc (Figure 3.3). In contrast, in the anterior pituitary a much smaller percentage of thyrotrophs, gonadotrophs and lactotrophs co-labeled with eYFP; TSH^{+ve};eYFP^{+ve} (31.7%, ±4.2, n = 3), LH^{+ve};eYFP^{+ve} (38.2%, ±7.1, n = 3) and PrL^{+ve};eYFP^{+ve} (34.6%, ±2.4, n = 3) (Figure 3.3). Similarly, the percentage of somatotrophs and corticotrophs that co-labeled with eYFP was less than in the melanotroph population, however it was more than other AP endocrine cells; GH (51.9%, ±2.0 n = 2) and ACTH (55.4%, ±3.6, n = 3) (Figure 3.3). This fate mapping data reveals that cells in which *Nkx3.1^{Cre}* is active will go on to produce endocrine cells in the anterior and intermediate lobes, but to varying levels.

A


Nkx3.1Cre/+;R26ReYFP/+
PL
IL
AL
DAPI
eYFP/GH
DAPI
eYFP/TSH
DAPI
eYFP/PrL
DAPI
eYFP/LH
DAPI
eYFP/POMC

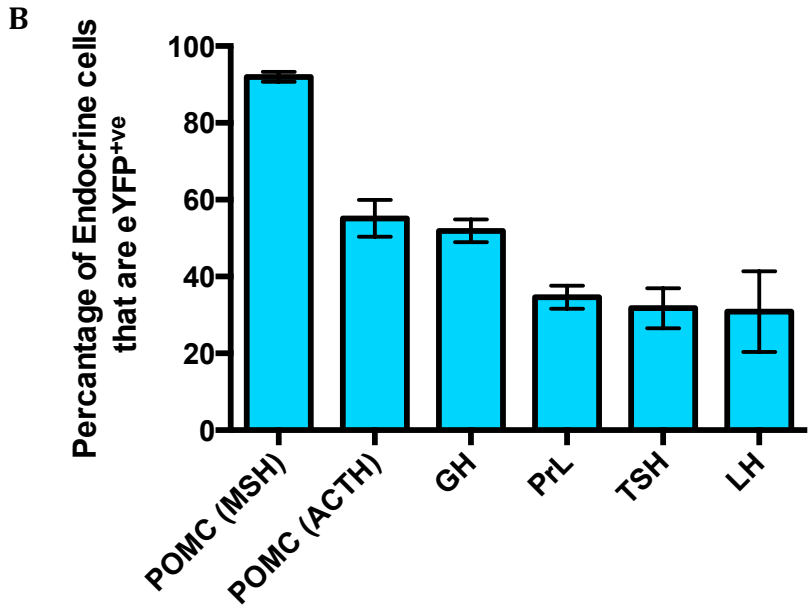


Figure 3.3: *Nkx3.1*^{Cre} fate mapping in all endocrine cell types.

A: Immunofluorescence stain for eYFP (green), hormone (red) counterstained with DAPI (blue) at 18.5dpc. eYFP expression is highest in the future IL, but is also scattered throughout the parenchyma in *Nkx3.1*$^{Cre/+}$*;R26R*$^{eYFP/+}$ embryos. Each individual endocrine cell type co-localises with eYFP to different extents, with the highest co-localisation occurring in the future IL. POMC staining represents MSH in the IL and ACTH in the AP. Each image represents half of one 18.5dpc pituitary, viewed in a coronal orientation (see Figure 1.3, 1.4 for schematic representation). Scale = 100µm. **B:** Proportion of each endocrine cell population that is eYFP^{+ve} at 18.5dpc as a percentage of each population. POMC (MSH) 92.0% (±1.8, n = 3), POMC (ACTH) 55.4% (±3.6, n = 3), GH 51.9% (±2.0, n = 2), PrL 34.6% (±2.4, n = 3), TSH 31.7% (±4.2, n = 3) and LH 38.2 (±7.1, n = 3).

3.2.3 *FoxG1^{Cre}* activity prolife

The second Cre driver explored as a means to conditionally delete *Sox2* is *FoxG1^{Cre}*, which has been reported to be active throughout the RP from at least 9.5dpc (Wang et al., 2010). To identify the activity profile of this Cre-driver, *FoxG1^{Cre}* mice were crossed with a Rosa26 Reporter eYFP strain (Srinivas et al., 2001). In embryos carrying both the Cre driver and reporter, at 10.5dpc all cells of are eYFP^{+ve} (n = 1) (Figure 3.2, 3.4). At this stage no cells of the overlying VD are eYFP^{+ve}, however by 12.5dpc *FoxG1^{Cre}* has become active in the VD, with some eYFP^{+ve} cells starting to appear (Figure 3.4). This VD expression may be due to the mice not being on a pure 129/SvJ background (Hébert & McConnell., 2000). These data suggest that, when combined with the *Sox2* conditional mutation, *Sox2* will be deleted throughout the RP from as early as 10.5dpc, however it will also be deleted in a salt and pepper fashion in the VD from 12.5dpc. Therefore any phenotype that results could be affected by partial loss of SOX2 in the VD. Due to the widespread activity of *FoxG1^{Cre}* at 10.5dpc in RP, by 18.5dpc all cells of the AP are eYFP^{+ve} (Figure 3.4). This activity is prior to the start of endocrine cell differentiation at 11.5dpc - 13.5dpc. Thus if SOX2 is required for endocrine cell differentiation, the subsequent global deletion may result in the complete loss of terminally differentiated endocrine cells.

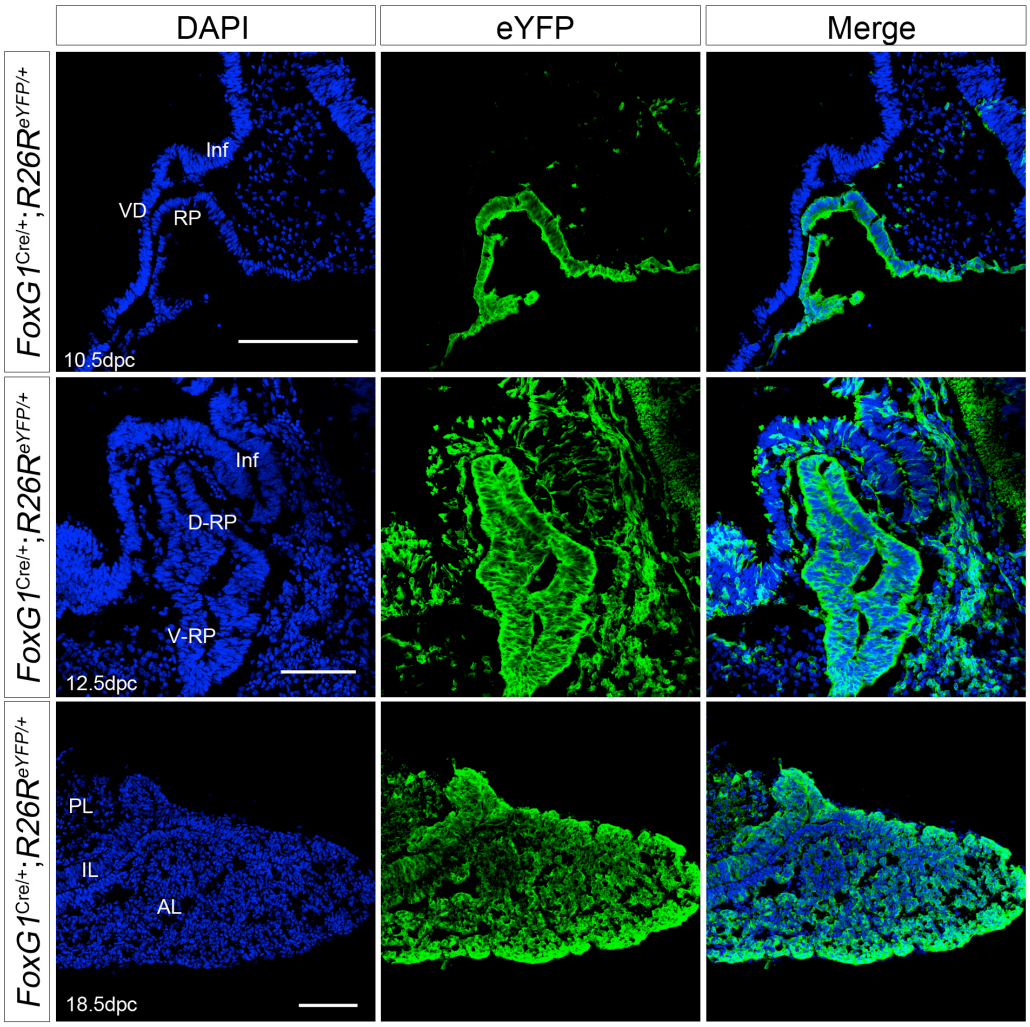


Figure 3.4: Activity profile of the *FoxG1^{Cre}* allele.

Immunofluorescence stain for eYFP (green), counterstained with DAPI (blue). eYFP is expressed in all cells in RP at 10.5dpc and is therefore retained in all cells at 12.5dpc and 18.5dpc in *FoxG1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos. eYFP expression is not observed in the VD at 10.5dpc, however subsequently it becomes upregulated in 12.5dpc embryos, in addition to becoming expressed in the surrounding mesenchyme. 10.5dpc and 12.5dpc sections are orientated sagittally; 18.5dpc sections are orientated coronally and represent half of one pituitary (see figure 1.3 and 1.4 for schematic representation). VD = Ventral Diencephalon, RP = Rathke's Pouch, Inf = Infundibulum, D-RP = Dorsal Rathke's, V-RP = Ventral Rathke's Pouch Pouch, PL = Posterior Lobe, IL = Intermediate Lobe and AL = Anterior Lobe. Scale = 100µm.

3.3 Discussion

The experiments carried out in this chapter aimed to investigate the properties of two different Cre drivers chosen to conditionally delete *Sox2* in RP. The different activity profiles of the Cre drivers would allow a comparison of the consequences of deleting *Sox2* in different spacio-temporal patterns, which might help to elucidate any role of SOX2 and to identify underlying mechanisms.

Birthdating studies have shown all RP progenitors proliferate up until 10.5dpc. Thereafter progenitors exit the cell cycle between 11.5dpc and 13.5dpc and begin to differentiate into each of the anterior pituitary endocrine cell lineages (Davis et al., 2011). At 10.5dpc all cells of RP are SOX2^{+ve}. The data obtained using *Nkx3.1^{Cre}* reveal a few eYFP^{+ve};SOX2^{+ve} cells at 10.5dpc, however by 12.5dpc the number had markedly increased. Consequently *Sox2* should be deleted in RP progenitors during the phase of cell cycle exit when using *Nkx3.1^{Cre}*. Conversely *FoxG1^{Cre}* is ubiquitously active in RP from 10.5dpc and possibly earlier (Wang et al., 2010). This should result in *Sox2* deletion during the proliferative stage of RP development prior to 10.5dpc and prior to cell cycle exit. One of the main advantages to using these two Cre drivers should be the ability to delete *Sox2* in two temporally specific patterns. This will allow for the analysis of the role of SOX2 both during the proliferative phase of RP development and just prior to differentiation.

The second advantage to using two Cre drivers is their different spatial patterns of activity. Lineage tracing using either *Sox2^{CreERT2}* or *Sox9^{CreERT2}* as inducible Cre drivers has revealed that the SOX2^{+ve} progenitors prior to or upon the start of differentiation give rise to all the endocrine cells of the anterior and intermediate lobes (Rizzoti et al., 2013). While strictly speaking not lineage tracing, the data shown here indicates that due to the early onset of *FoxG1^{Cre}* activity all endocrine cells are eYFP^{+ve} at 18.5dpc. This should allow the consequences of *Sox2* deletion on endocrine cell differentiation to

be studied and identify whether SOX2 is necessary for the differentiation of all or a subset of endocrine cell types. *Nkx3.1^{Cre}* however, is not active throughout the entire RP at any stage, resulting in varying levels of hormone^{+ve};eYFP^{+ve} cells at 18.5dpc. In the developing AP, *Nkx3.1^{Cre}* is active in the ventral regions of RP in a salt and pepper fashion. At this stage of development endocrine cell cycle exit varies depending on cell type, 11.5dpc-12.5dpc for gonadotrophs and thyrotrophs to 11.5-13.5dpc for corticotrophs and somatotrophs (Davis et al., 2011). As a result, if this Cre-driver acts similarly on *Sox2$^{fl/fl}$* as it does on *R26ReYFP, Sox2* will be deleted in a proportion of each endocrine cell type during the period of cell cycle exit and in addition, *Sox2* will be deleted in each cell type to a different extent. This may result in a greater reduction in endocrine cell number than is predicted based solely on the expression of the eYFP reporter.

Birthdating studies have shown that MSH cells exit the cell cycle between 12.5-14.5dpc, later than AP endocrine cells (Davis et al., 2011). eYFP expression in the dorsal region of RP reveals that *Nkx3.1^{Cre}* is ubiquitously active in this region at 12.5dpc and gives rise to over 90% of POMC^{+ve} MSH cells at 18.5dpc. As a consequence of this, it should be possible to use *Nkx3.1^{Cre}* to delete *Sox2* just prior to the exit of the cell cycle in the dorsal region of RP. This may allow the role of SOX2 to be determined in the cycling progenitors of cells that give rise to only one endocrine cell type.

Nkx3.1^{Cre} offers additional advantages to the study of the role of SOX2 in RP development. The mosaic deletion pattern, along with the presence of a reporter allele, allows the fate of a subset of *Sox2* deleted cells to be followed in the developing AL. While SOX2 must function cell autonomously, it has been shown to drive neurogenesis in the hippocampus in a non-cell autonomous fashion, via its direct transcriptional regulation of *Shh* transcription. *Sox2* conditional deletion in these cells downregulates SHH expression and therefore lowers SHH singling, thus impeding neurogenesis in the surrounding tissue (Favaro et al., 2009). *SoxB1* genes have also been identified as non-cell autonomous regulators of pituitary development via

the transcriptional regulation of *Shh* expression in the VD (Zhao et al., 2012). In this case, SHH aids the development of the infundibulum and RP (Zhao et al., 2012). Conversely, conditional deletion studies show the cell autonomous roles of SOX2 and SOX3 in the lungs and testis (Laronda et al., 2011; Que et al., 2009) . *Sox3* conditional deletion using *VasaCre* prevents spermatogonal differentiation in prepubertal mice, a phenotype similar to that seen in *Sox3* null mice, indicating the intrinsic, cell autonomous role SOX3 plays in these cells (Laronda et al., 2011). Through the use of a reporter allele, it should be possible to trace and compare the development of cells deleted for *Sox2* and those that still retain it (non-eYFP) cells. One caveat to this is one must assume the Cre recombinase excises the STOP codon in the reporter allele with the same efficiency as it excises conditional $Sox2^{fl/fl}$. Varying degrees of Cre recombinase efficiency has been observed in other studies (Liu et al., 2012), however the consistency in deletion and eYFP expression will identify if excision is equally efficient between $R26R^{eYFP}$ and $Sox2^{fl/fl}$. Nevertheless this should address whether SOX2 acts only in a cell autonomous fashion, e.g. *Sox2* deletion has no affect on the remaining population of cells of that lineage or any other lineage, or in a non-cell autonomous fashion, i.e. *Sox2* deletion in a subset of cells directly affects the development of surrounding cells.

The mosaic activity of $Nkx3.1^{Cre}$ may also be a drawback. If *Sox2* is not deleted throughout RP it might be difficult to ascertain its role in all RP progenitors. In contrast, $FoxG1^{Cre}$ is active in the hypophyseal placode at 8.5dpc (Wang et al., 2011) and by 10.5dpc, as shown here, 100% of $SOX2^{+ve}$ cells express, or must have expressed Cre recombinase (in comparison to 68.6% when using $Nkx3.1^{Cre}$). These results suggest using $FoxG1^{Cre}$ will allow us to identify the earlier, global role of SOX2 in RP development, prior to the onset of dorsal restriction.

Previous studies have indicated that the genetic background on which $FoxG1^{Cre}$ is bred makes a considerable difference to its pattern of activity (Hébert & McConnell., 2000). The pattern of activity induced by $FoxG1^{Cre}$ on

a 129/SvJ and Swiss Webster background is the most consistent, while BALB/c is the least consistent (Hébert & McConnell., 2000). In our study, *FoxG1^{Cre}* was not bred onto a pure 129/SvJ background (it was imported on a mixed background and it would have taken too many generations of backcrossing to do so). It is assumed, therefore, that this is the reason it showed ectopic recombination in the VD/infundibulum, however this did not occur until 12.5dpc.

It is known that the infundibulum directly influences RP development through the secretion of BMP4 and FGF8/10 (Davis et al., 2011; Norlin et al., 2000; Ericson et al., 1998; Treier et al., 1998). The results shown here suggest that the use of the *FoxG1^{Cre}* driver may lead to the deletion of *Sox2* in a few cells of the infundibulum, which could mildly affect its development. Disruption of the infundibulum could, as a consequence, impair the development of RP, as seen with *Sox3* mutants, resulting in a phenotype not wholly derived from the deletion of *Sox2* in RP (Rizzoti et al., 2004). Analysis of the expression of TF that are directly regulated by FGF8/10 and BMP4 secretion from the VD will help to determine whether this is occurring (See chapter 4).

3.3.1 Conclusions

In conclusion, *Nkx3.1^{Cre}* and *FoxG1^{Cre}* will provide alternative patterns of *Sox2* deletion in RP. This provides an advantage, as we can investigate the role of SOX2 in different temporal and spatial contexts. We can delete *Sox2* globally and extremely early on in RP development, prior to cell cycle exit using *FoxG1^{Cre}*. In addition we can also investigate its role specifically in the most dorsal region, in cells that will give rise to the IL and in a mosaic pattern in the AL, with *Nkx3.1^{Cre}*.

4. Analysis of the role of SOX2 in the developing pituitary

The different activity profiles of *Nkx3.1^{Cre}* and *FoxG1^{Cre}* provide alternative temporal and spatial patterns to conditionally delete *Sox2 in vivo*. In this chapter, the phenotypes that result from deletion using each of these Cre drivers will be described and analysed. The relevance of these results will then be discussed with respect to previously published data and hypotheses raised in earlier chapters.

4.1 Introduction

The proliferative capacity of cells in the pituitary varies significantly between development and adulthood. Early ubiquitous SOX2 expression in RP coincides with a high level proliferation (Fauquier et al., 2008). As development proceeds, proliferative potential is reduced, but it remains mainly associated with SOX2 expression, in the dorsal region of RP. (Fauquier et al., 2008; Rizzoti & Pires unpublished). Subsequently, by 18.5dpc only 1% of SOX2^{+ve} cells incorporate BrdU following a 1-hour pulse, compared to 80% at 12.5dpc (Fauquier et al., 2008). The proliferative capacity of the adult SOX2^{+ve} population remains very low, however it increases significantly following Gdx or Adx (Rizzoti et al., 2013). This indicates that these cells retain the capacity to proliferate in response to physiological demand.

The association between SOX2 and proliferation is similar in a number of other tissues throughout development, including the CNS. Initially expressed throughout the neurepithelia and neural tube proliferative SOX2^{+ve} progenitors become progressively restricted to the ventral region of the neural tube as development progresses, similar to what is observed in RP (Ellis et al., 2004; Fauquier et al., 2008). Nevertheless, in adult mice, the neurogenic region of the lateral ventricle maintains a population of SOX2^{+ve} cells that remain proliferative (Ferri et al., 2004). Some of these are NSC,

although transit-amplifying progenitors also express SOX2 and can replicate. Downregulation of SOX2 however, is necessary for these cells to become neuroblasts (López-Juárez et al., 2012).

Cells expressing *Sox2* in the eye share a similar proliferative profile as in the CNS and pituitary. SOX2^{+ve} proliferative progenitors are found primarily in the central region of the optic cup in development (Matsushima et al., 2011). Proliferation in these cells is lost following *Sox2* deletion, moreover *Sox2* hypomorphic mice have a small eye defect (Matsushima et al., 2011; Taranova et al., 2006). Conversely in the adult eye, SOX2 is expressed in Müller glial cells that retain a neurogenic capacity and are largely quiescent, as in the brain (Surzenko et al., 2013). *Sox2* deletion results in loss of the glial cells population as these cells re-enter the cell cycle through the downregulation of *p27kip1* (Surzenko et al., 2013).

In the embryonic cochlea an increase in the number of SOX2^{+ve} cells through βcatenin overexpression is coupled with an increase in Cyclin D1 expression and proliferation (Jacques et al., 2013). An increase in Cyclin D1 expression, coupled with an increase in proliferation is also observed in the adult respiratory epithelium upon SOX2 overexpression (Tompkins et al., 2011). These observations, derived from other SOX2^{+ve} progenitor populations, imply that the role of SOX2 in RP progenitors may also be, at least in part, to maintain proliferation. Deletion of *Sox2* early in RP using *FoxG1^{Cre}* should provide a test of this hypothesis.

The aim of this chapter was to analyse the effect that conditional *Sox2* deletion has on the embryonic development of the pituitary. Both *Nkx3.1^{Cre}* and *FoxG1^{Cre}* drivers were used in parallel, however the latter was used primarily in the analysis of early TF expression due to its early and ubiquitous activity profile in RP. *Nkx3.1^{Cre}* activity starts later and is restricted, so was used to investigate later aspects of SOX2 function.

4.2 Results

4.2.1 Morphological analysis of Rathke's Pouch following *Sox2* deletion

At 10.dpc *Sox2* is expressed throughout RP (Figures 4.1, 4.2). To assess the effects of partial and ubiquitous *Sox2* deletion in RP, $Sox2^{fl/fl}$ mice were bred with $Sox2^{fl/+};Nkx3.1^{Cre/+}$ and $Sox2^{fl/+};FoxG1^{Cre/+}$ mice. SOX2 expression is almost unaffected at 10.5dpc in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos (Figure 4.1). This is coupled with no morphological defects and this matches the small number of cells in which $Nkx3.1^{Cre}$ is active in at this stage of development. $Nkx3.1^{Cre}$ activity is markedly increased between 10.5dpc and 12.5dpc (Figure 3.1). This increase in activity is mirrored in the reduction of SOX2 expression at 12.5dpc in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos where only a small number of cells still express SOX2 in the dorsal region (Figure 4.1). Despite the loss of the protein, there are no clear morphological abnormalities at this stage of development (Figure 4.1). Conversely by 14.5dpc morphological defects start to appear in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos. The dorsal region has become a thinner more rounded disrupted epithelium (Figure 4.1). The number of $SOX2^{+ve}$ cells is clearly decreased compared to control embryos and appears lower than at 12.5dpc (Figure 4.1).

In contrast to $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos, SOX2 is completely downregulated in the RP of $Sox2^{fl/fl};FoxG1^{Cre/+}$ embryos at 10.5dpc (Figure 4.2). Nevertheless this is not coupled to any morphological defects at this stage (Figure 4.2). By 12.5dpc however, RP is severely hypomorphic and has failed to pinch off from the underlying oral ectoderm. This is in stark contrast to control $Sox2^{fl/+};FoxG1^{Cre/+}$ embryos, in which RP is a defined structure at 12.5dpc (Figure 4.2). At 14.5dpc the morphological defects are even more pronounced as RP has now pinched off from the oral ectoderm, and is extremely aberrant (Figure 4.2). Despite the pinching off that occurs at 14.5dpc, the sphenoid bone fails to close up at 14.5dpc and 16.5dpc (Figure 4.3, 4.20). The tissue that extends into the gap between the un-fused sphenoid bone is initially comprised of rostral-tip thyrotrophs (Figure 4.3).

Notwithstanding this, by 16.dpc the tissue is overwhelmingly hormone negative (Figure 4.3). *FoxG1*^{Cre} is active throughout the oral ectoderm at 10.5dpc and co-localises with SOX2 in this region. Deletion of *Sox2* in the oral ectoderm may compromise sphenoid bone closure (Figure 2.4).

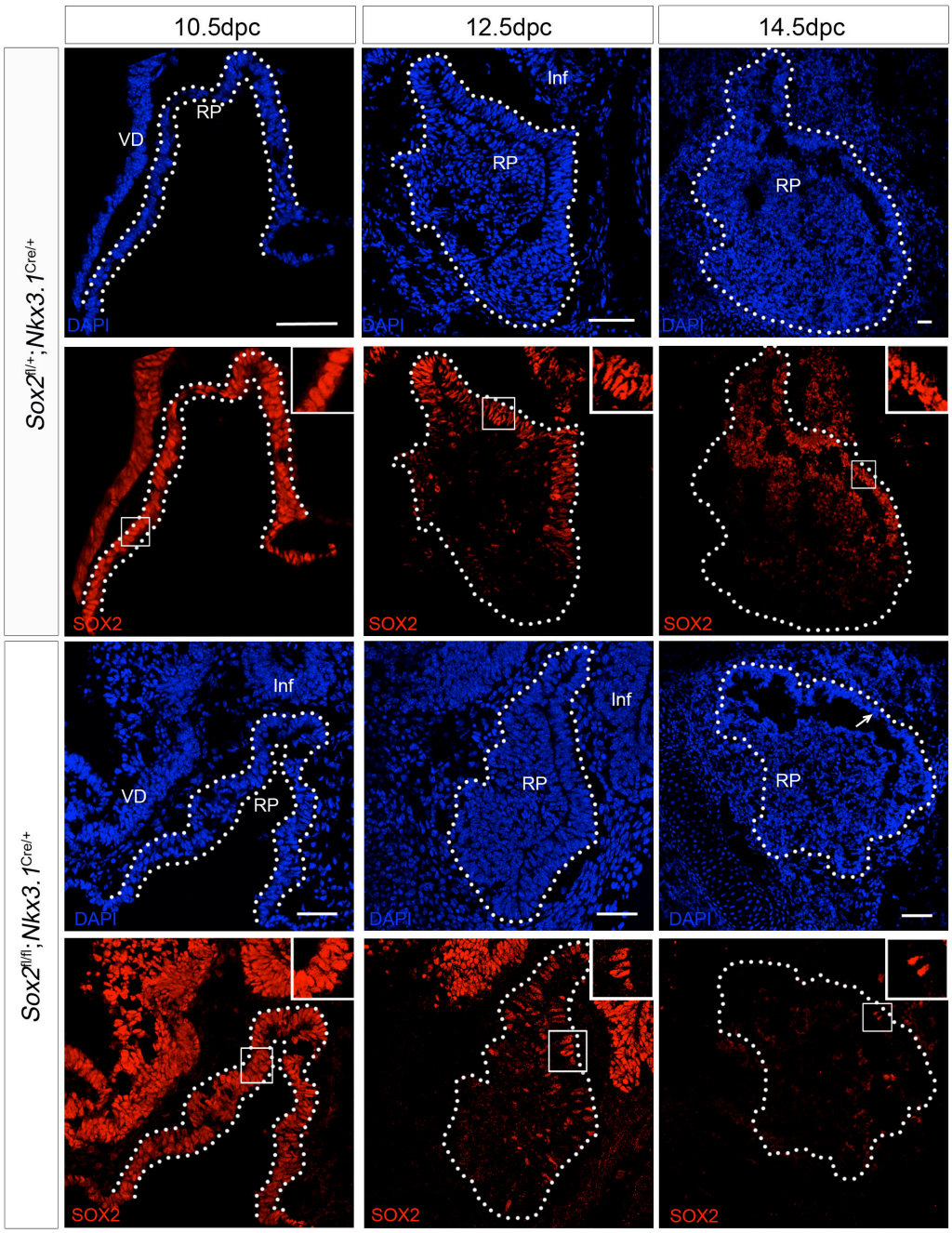


Figure 4.1: Conditional deletion of *Sox2* using *Nkx3.1^{Cre}*.

Immunofluorescence for SOX2 (red), counterstained with DAPI (blue) at 10.5dpc, 12.5dpc and 14.5dpc. Nuclear SOX2 (inset) is found throughout RP at 10.5dpc in control *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$* embryos and is only lost in a handful of cells in mutant *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos. SOX2 is also observed throughout the VD and infundibulum at 10.5dpc. SOX2 becomes restricted to the dorsal side of RP by 12.5dpc and maintained there at 14.5dpc in control *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$* embryos. There is a dramatic reduction in the number of SOX2 positive cells in RP of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos at 12.5dpc and 14.5dpc in addition to a thinning of the dorsal region of RP at 14.5dpc (arrow). Morphological differences can be observed by 14.5dpc. All sections are oriented sagittally (see figure 1.3 for schematic representation). RP = Rathke's Pouch, Inf = Infundibulum. Scale = 50µm.

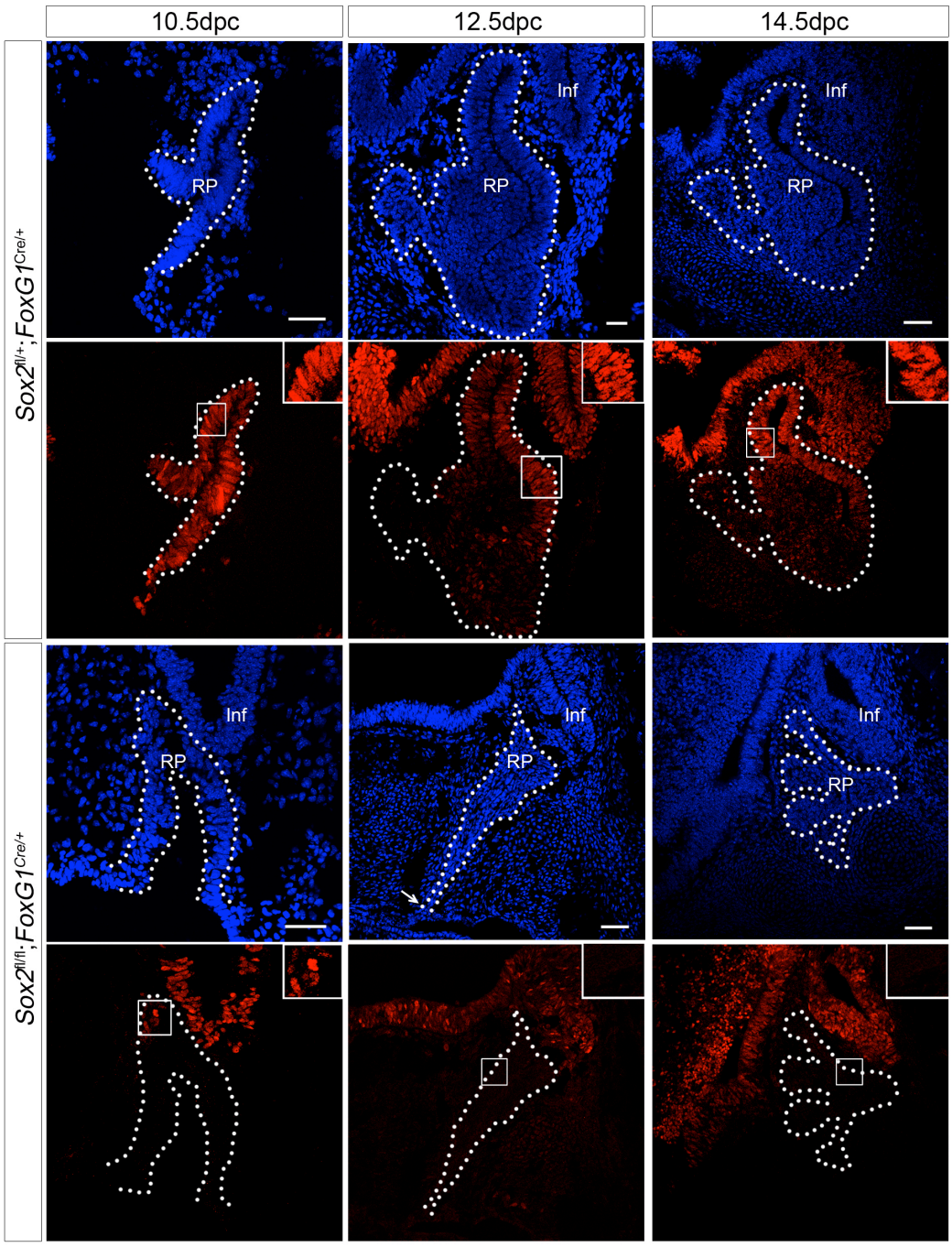


Figure 4.2: Conditional deletion of *Sox2* using *FoxG1^{Cre}*.

Immunofluorescence for SOX2 (red), counterstained with DAPI (blue) at 10.5, 12.5 and 14.5dpc. Nuclear SOX2 (inset) is found throughout RP at 10.5dpc in *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos, becoming progressively dorsally restricted at 12.5dpc and 14.5dpc. In mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos SOX2 is almost completely downregulated at least from 10.5dpc. No obvious morphological defects are observed until 12.5dpc in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryo, when an hypoplastic RP failing to pinch off from the underlying oral ectoderm is observed (arrow). Detachment from the oral ectoderm is however achieved by 14.5dpc. All sections are orientated sagittally (see figure 1.3 for schematic representation). RP = Rathke's Pouch, Inf = Infundibulum. Scale = 50μm.

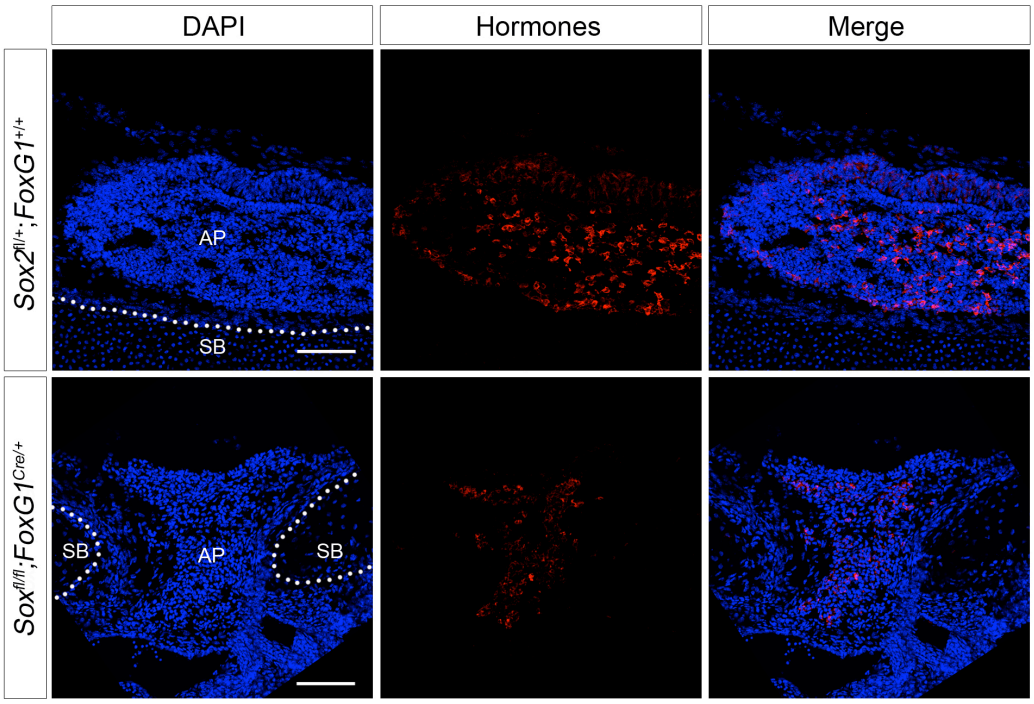


Figure 4.3: Failure of sphenoid bone closure following *Sox2* deletion using *FoxG1*^{Cre}. Immunofluorescence for all AL hormones (GH, PRL, TSH, ACTH and LH, (see materials and methods) red), counterstained with DAPI (blue) at 16.5dpc. In mutant *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$ embryos the sphenoid bone fails to fuse at the midline. This results in the displacement of the residual AL, identified by the presence of AL hormone positive cells displaced ventrally in to the resulting gap. Sections orientated coronally. AP = Anterior Pituitary, SB = Sphenoid Bone. Scale = 100µm.

4.2.2 Morphological analysis of the adult pituitary following *Sox2* deletion using *Nkx3.1^{Cre}*

In adult WT mice an epithelium of SOX2^{+ve} cells line the lumen of the pituitary (Fauquier et al., 2008). SOX2^{+ve} cells that line the lumen in the pituitary of adult *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* mice are extremely sparse (Figure 4.4A). The epithelium is only ever one cell thick and breaks in SOX2^{+ve} cells are more common than a linear arrangement (Figure 4.4A). This likely represents the result of the abnormal development of RP leading to a hypoplastic pituitary, thinner lining of the cleft and more sparsely distributed SOX2^{+ve} cells. Developmentally the dorsal region of RP gives rise to the IL in the adult pituitary (for review see Lovell-Badge & Rizzoti., 2005). Lineage tracing indicates that *Nkx3.1^{Cre}* is most active, and therefore *Sox2* deletion most efficient, in the dorsal region of RP at 12.5dpc (Figures 3.1). POMC staining further reveals that the most severe phenotype associated with *Sox2* deletion using *Nkx3.1^{Cre}* is the reduction of the IL (Figure 4.4B).

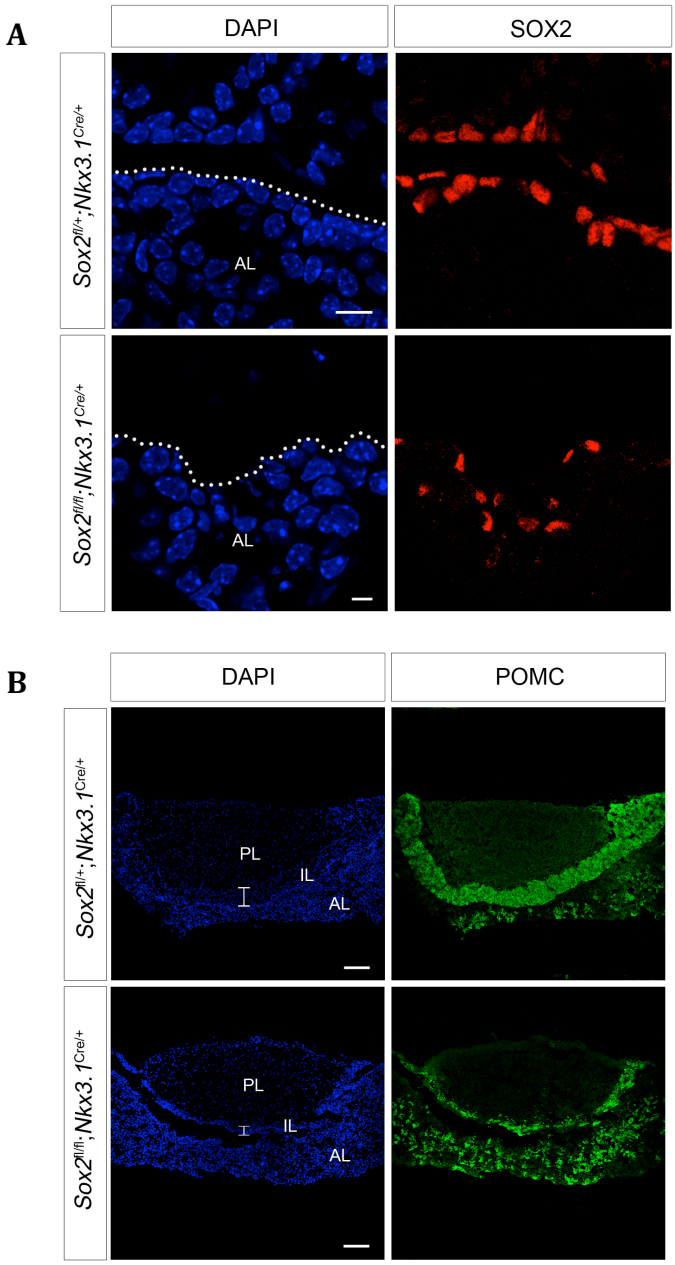


Figure 4.4: Adult pituitary phenotype following *Sox2* deletion using *Nkx3.1*^{Cre}.

A) *Sox2* expression in cells lining the lumen of adult pituitaries**.** Immunofluorescence for SOX2 (red), counterstained with DAPI (blue) reveals. SOX2^{+ve} cells present in the epithelium lining the lumen of *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ pituitaries. This layer is severely disrupted in *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ pituitaries. AP = anterior pituitary. Scale = 10µm. **B)** POMC expression in adult pituitaries. Immunofluorescence for POMC (green), counterstained with DAPI (blue). The IL in *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ mice is severely reduced in size compared to control *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ mice (bracket). Accordingly expression of POMC in IL melanotrophs is reduced, while expression in AL corticotrophs is not obviously affected. All sections are orientated coronally. AL = Anterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 100µm.

4.2.3 Progenitor cell proliferation following *Sox2* deletion

To explore whether SOX2 regulates proliferation in RP progenitors, and whether this accounts for the hypoplastic phenotype seen after Cre-mediated deletion of *Sox2*, proliferation rates were analysed by BrdU incorporation. A one-hour pulse was performed on pregnant females at 12.5dpc as SOX2 loss was observed at this stage of embryo development when using each of the two Cre drivers (Figure 4.1, 4.2). In the $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos, the number of proliferating cells was first analysed in the whole RP, revealing only a small decrease in the number of cells incorporating BrdU. However, $Nkx3.1^{Cre}$ is predominantly active in the dorsal region of RP at 12.5dpc (Figure 3.1), with deletion only occurring in this same region (Figure 4.1). When BrdU incorporation was only quantified in the dorsal region of 12.5dpc RP, this revealed a large decrease in the percentage of $BrdU^{+ve}$ cells to $DAPI^{+ve}$ nuclei in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ (11.3%, ±2.6, n = 3), compared to $Sox2^{fl/+};Nkx3.1^{Cre/+}$ embryos (19.6%, ±1.8, n = 3) (Figure 4.5A).

However, when $FoxG1^{Cre}$ was used to delete *Sox2*, the decrease in the number of $BrdU^{+ve}$ cells in RP was significant when comparing $Sox2^{fl/+};FoxG1^{Cre/+}$ embryos with $Sox2^{fl/fl};FoxG1^{Cre/+}$ embryos; 17.2% (±1.0, n = 3) compared to 7.7% (±2.0, n = 3) (Figure 4.5B). In this instance the number of BrdU cells in the whole RP was counted, as $FoxG1^{Cre}$ is active in all cells at 10.5dpc (Figure 3.2). These results show that loss of *Sox2* result in a significant reduction in RP progenitor proliferation, and subsequently the formation of a hypoplastic pituitary.

To further analyse this proliferative defect Cyclin D1 expression was examined. Cyclin D1 is a cell cycle checkpoint protein considered to regulate the transition between the G_1/S phase of the cell cycle (Sala & Calabretta., 1992). Along with Cyclin D2, Cyclin D1 is found in dorsal proliferating progenitors of RP (Figure 4.6) (Bilodeau et al., 2009). Control $Sox2^{fl/+};FoxG1^{Cre/+}$ embryos have a defined region of Cyclin D1 expression in

the dorsal side of RP (Figure 4.6). This defined region of Cyclin D1^{+ve} proliferating progenitors is lost in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos (Figure 4.6). Loss of Cyclin D1 may cause cell cycle arrest at the G_1/S checkpoint, preventing progenitor proliferation.

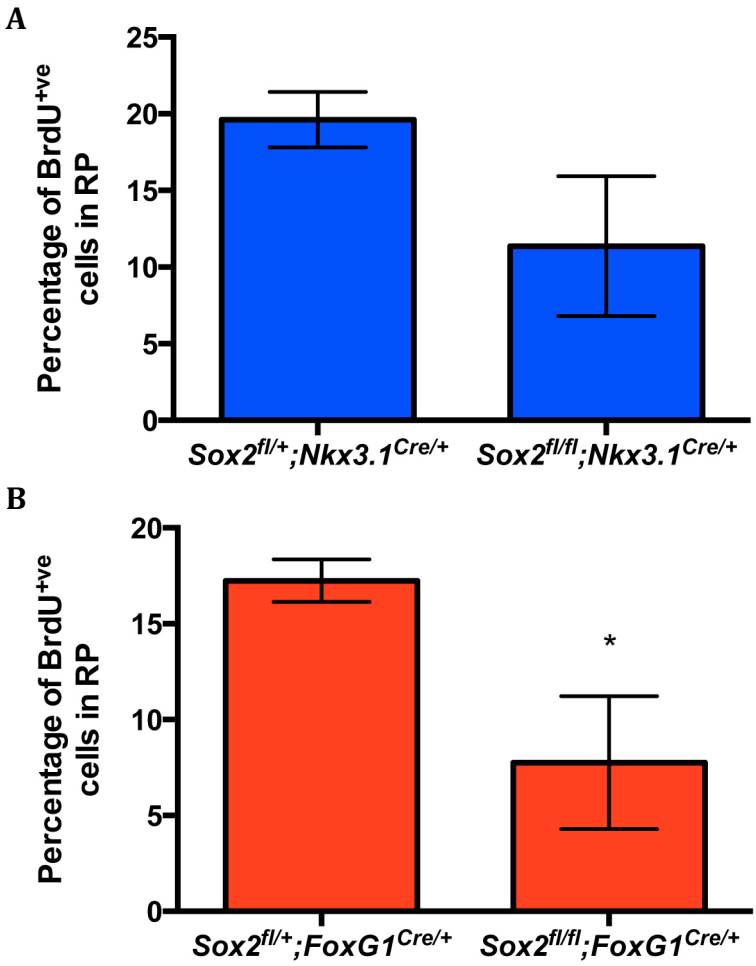


Figure 4.5: BrdU incorporation in RP following *Sox2* deletion

A: BrdU incorporation one hour after injection is reduced in *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos 11.3% (±2.6, n = 3), compared to control *Sox2*$^{fl/+}$;*Nkx3.1*$^{Cre/+}$ embryos 19.61% (±1.8, n = 3). Only cells in the dorsal region of RP were counted at this is where *Nkx3.1*^{Cre} is active, however the reduction is not significant. **B:** BrdU incorporation is significantly reduced in *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$ embryos at 7.7% (±2.0, n = 3), compared to control *Sox2*$^{fl/+}$;*FoxG1*$^{Cre/+}$ embryos at 17.2% (±1.09, n = 3). BrdU incorporation was counted over the whole RP in 'B' as *FoxG1*^{Cre} is active throughout the RP from 10.5dpc.

To address the role of cell cycle checkpoint markers in more detail, CDKI p27kip1 expression was analysed. In control embryos p27kip1 is expressed in the ventral region of RP at 14.5dpc in cells that have begun to differentiate (Bilodeau et al., 2009). No expression is observed in the dorsal region of RP where SOX2 is expressed suggesting they have a mutually exclusive pattern of expression in RP at 14.5dpc (Figure 4.7). Upon *Sox2* deletion the region of p27kip1 expression in the ventral region of RP is enlarged. Significantly, p27kip1 is ectopically expressed in the dorsal region of RP, though it is never ectopically co-expressed in any cells that fail to delete *Sox2* (Figure 4.7). Therefore *Sox2* deletion is also associated with ectopic up-regulation of p27kip1, which would result in cell cycle arrest, thus lowering proliferation.

Lowered BrdU incorporation, as a consequence at least in part of Cyclin D1 downregulation and p27kip1 up-regulation results in a decrease in proliferation, but a hypoplastic phenotype could also result from increased apoptosis. TUNEL staining in RP of control 12.5dpc embryos reveals there is very little programmed cell death occurring at this time (Figure 4.8), and this is not increased in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos. This suggests that apoptosis does not contribute to the hypoplasia (Figure 4.8). Conversely, a large number of $TUNEL^{+ve}$ apoptotic cells is observed ventrally in $Sox2^{fl/fl};FoxG1^{Cre/+}$ RP (Figure 4.8). It has been shown that as RP pinches off from the underlying oral ectoderm at 11.5dpc, there is a localised foci of apoptotic cells (Charles et al., 2005). The hypomorphic RP in $Sox2^{fl/fl};FoxG1^{Cre/+}$ embryos is still attached to the underlying oral ectoderm at 12.5dpc and this is where apoptotic cells are. The domain of apoptosis is however larger than in the equivalent 11.5dpc WT embryos (Charles et al., 2005), and expand into the ventral oral ectoderm, suggesting that some of the apoptosis occurring may be ectopic (Figure 4.8).

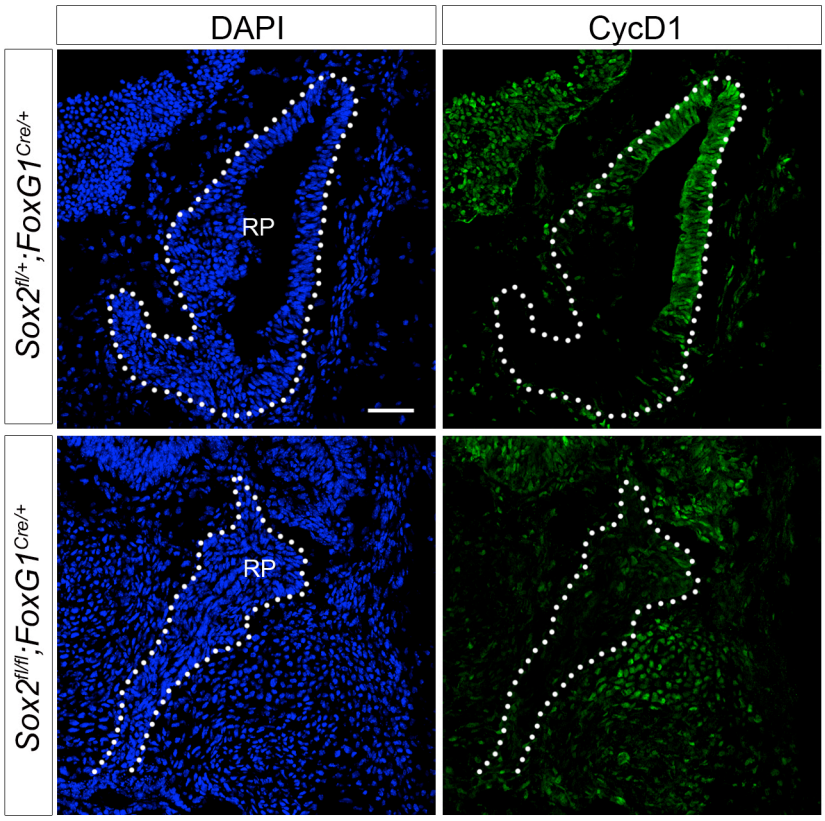


Figure 4.6: Cyclin D1 expression in 12.5dpc RP following *Sox2* deletion using *FoxG1*^{Cre}. Immunofluorescence for Cyclin D1 (green), counterstained with DAPI (blue) at 12.5dpc. Cyclin D1 is expressed throughout the dorsal region of RP in control *Sox2*$^{fl/+}$;*FoxG1*$^{Cre/+}$ embryos, but it is substantially reduced in the RP of *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$ embryos. All sections are orientated sagittally. RP = Rathke's Pouch. Scale = 100um.

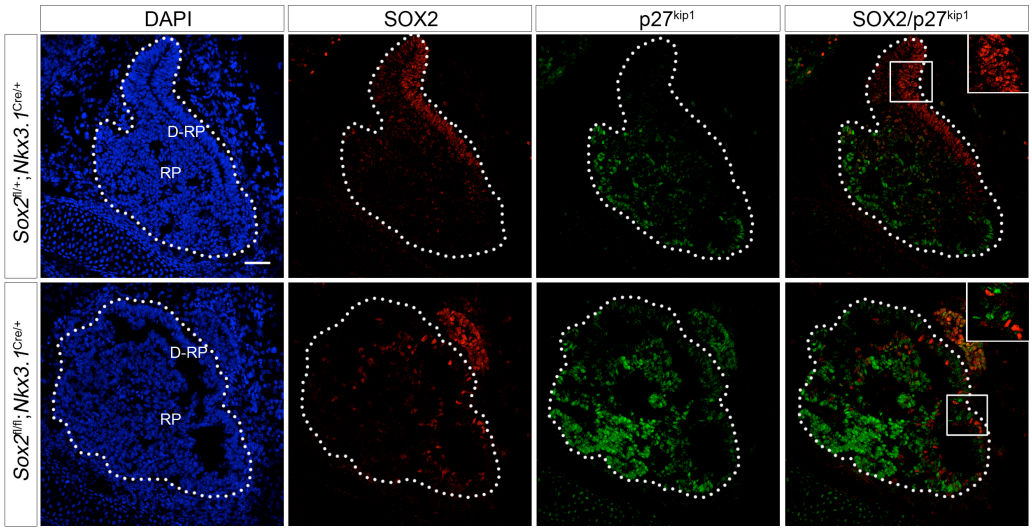


Figure 4.7: SOX2 and p27Kip1 expression in 14.5dpc RP following *Sox2* deletion using *Nkx3.1*^{Cre}.

Immunofluorescence for SOX2 (red) and p27kip1 (green), counterstained with DAPI (blue) at 14.5dpc. SOX2 is restricted to the dorsal side (D-RP and inset) of RP at 14.5dpc in control *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ embryos. Conversely p27kip1 is expressed ventrally. In *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos p27kip1 is ectopically expressed dorsally, however never in the few remaining SOX2^{+ve} cells (inset). All sections are orientated sagittally. RP = Rathke's Pouch, D-RP = Dorsal Rathke's Pouch. Scale 100µm.

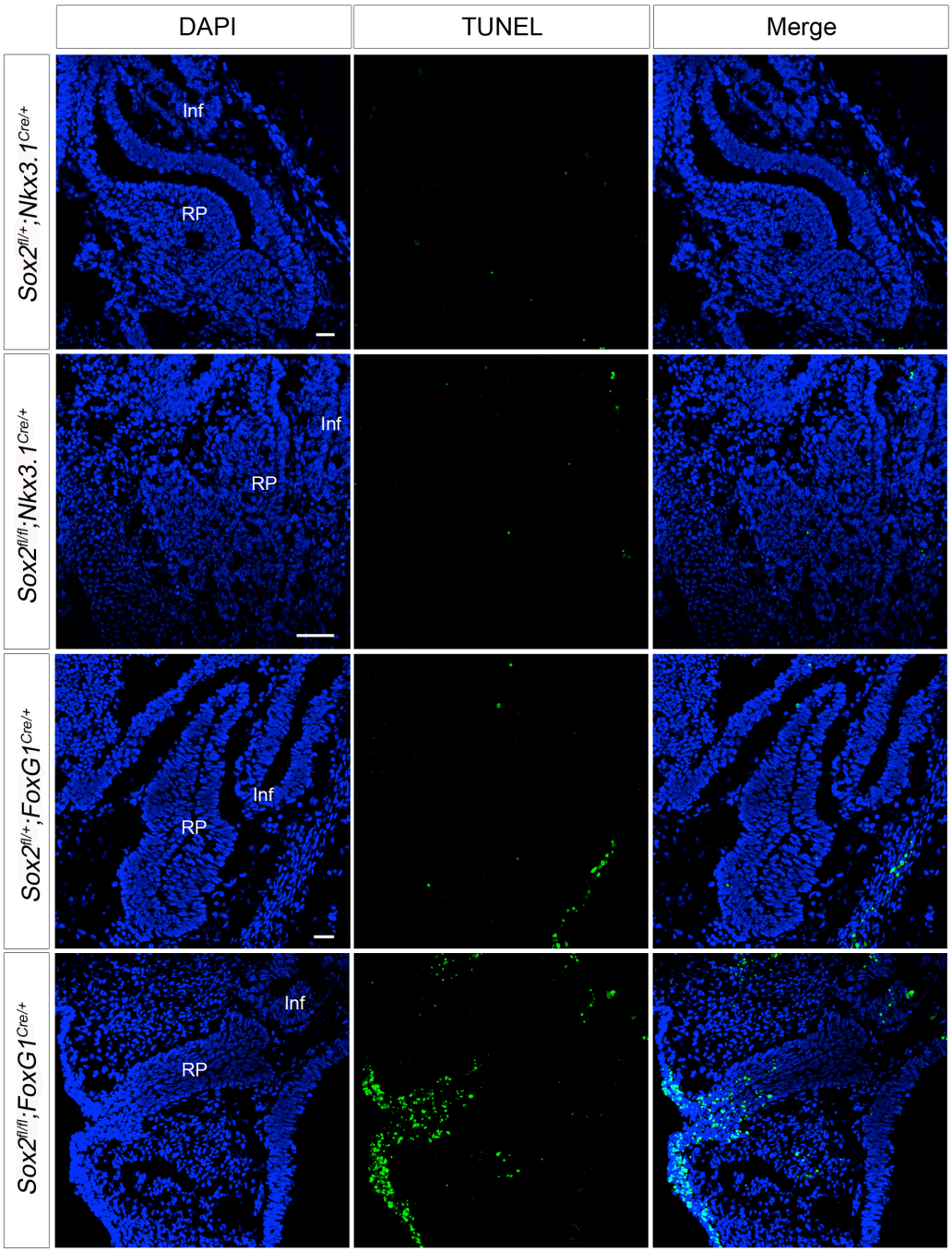


Figure 4.8: TUNEL staining in 12.5dpc RP following *Sox2* deletion.

TUNEL assay (green), counterstained with DAPI (blue) at 12.5dpc. Very little apoptosis is observed in RP at 12.5dpc as only two to three TUNEL^{+ve} cells are observed in control *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ and *Sox2*$^{fl/+}$*;FoxG1*$^{Cre/+}$ embryos. No increase in apoptosis is observed in *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos. Increased apoptosis is observed in *Sox2*$^{fl/fl}$*;FoxG1*$^{Cre/+}$ embryos where RP is pinching off from the underlying oral ectoderm, indicated by an a large number of TUNEL^{+ve} cells. No TUNEL^{+ve} cells are seen in the dorsal region of RP in *Sox2*$^{fl/fl}$*;FoxG1*$^{Cre/+}$ embryos. All sections are orientated sagittally. Inf = Infundibulum, RP = Rathke's Pouch. Scale = 50μm.

4.2.4 Early Rathke's Pouch transcription factor expression following *Sox2* deletion

Many TFs expressed in the early RP are known to regulate progenitor proliferation (for review see Kelberman et al., 2006). Null deletion of TFs such as *Isl1*, *Lhx3* and *Six6/3* result in hypoplastic pituitaries similar to the ones observed when *Sox2* is deleted using either Cre driver (Takuma et al., 1998; Sheng et al., 1997 Li et al., 2002). Analysis of early RP TF expression was carried out to identify any alteration.

ISL1 initially becomes restricted to RP from 9.5dpc and is ubiquitously expressed throughout RP at 10.5dpc in control embryos (Ericson et al., 1998) (Figure 4.9). *Sox2* deletion in mutant $Sox2^{fl/fl};FoxG1^{Cre/+}$ embryos has no effect on ISL1 expression (Figure 4.9). LHX3 expression also starts at around 9.5dpc, and is found throughout the pituitary by 16.5dpc, although with a gradient of expression higher in dorsal regions. ISL1 along with PITX1 has been shown to drive *Lhx3* expression in RP (Mullen et al., 2012). We therefore hypothesise that the undisrupted ISL1 expression should result in undisrupted LHX3 expression. At 10.5dpc SOX2 and LHX3 are co-expressed throughout RP in control embryos (Figure 4.10). At 12.5dpc SOX2 is becoming restricted to the dorsal region but LHX3 is still expressed in every cell of RP resulting in loss of co-expression ventrally (Figure 4.10). *Sox2* deletion using $FoxG1^{Cre}$ completely downregulates SOX2 but has no affect on LHX3 expression at 10.5dpc and 12.5dpc (Figure 4.10). LHX3 is still expressed in the ventral region of the mutant RP that is now the area pinching off from the oral ectoderm (Figure 4.10).

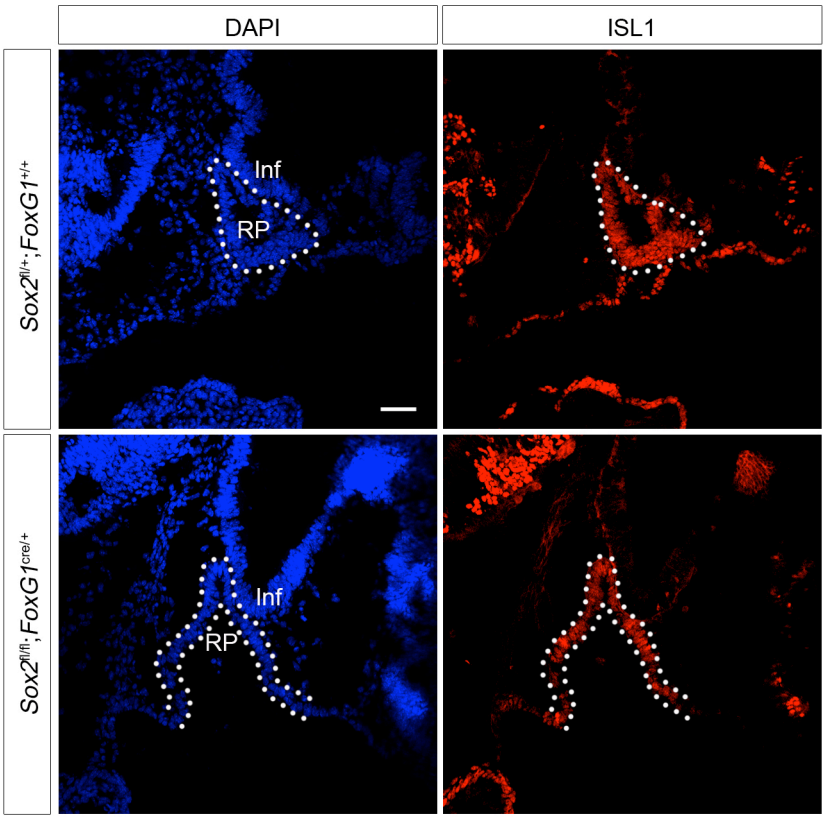


Figure 4.9: ISL1 expression at 10.5dpc in RP following *Sox2* deletion using *FoxG1*^{Cre}. Immunofluorescence for ISL1 (red), counterstained with DAPI at 10.5dpc. ISL1 is ubiquitously expressed in RP at 10.5dpc in control *Sox2*$^{f/+}$;*FoxG1*$^{+/+}$ embryos. ISL1 remains in all cells of RP in mutant *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$ embryos. All sections are orientated sagittally. Inf = Infundibulum, RP = Rathke's Pouch. Scale = 50μm.

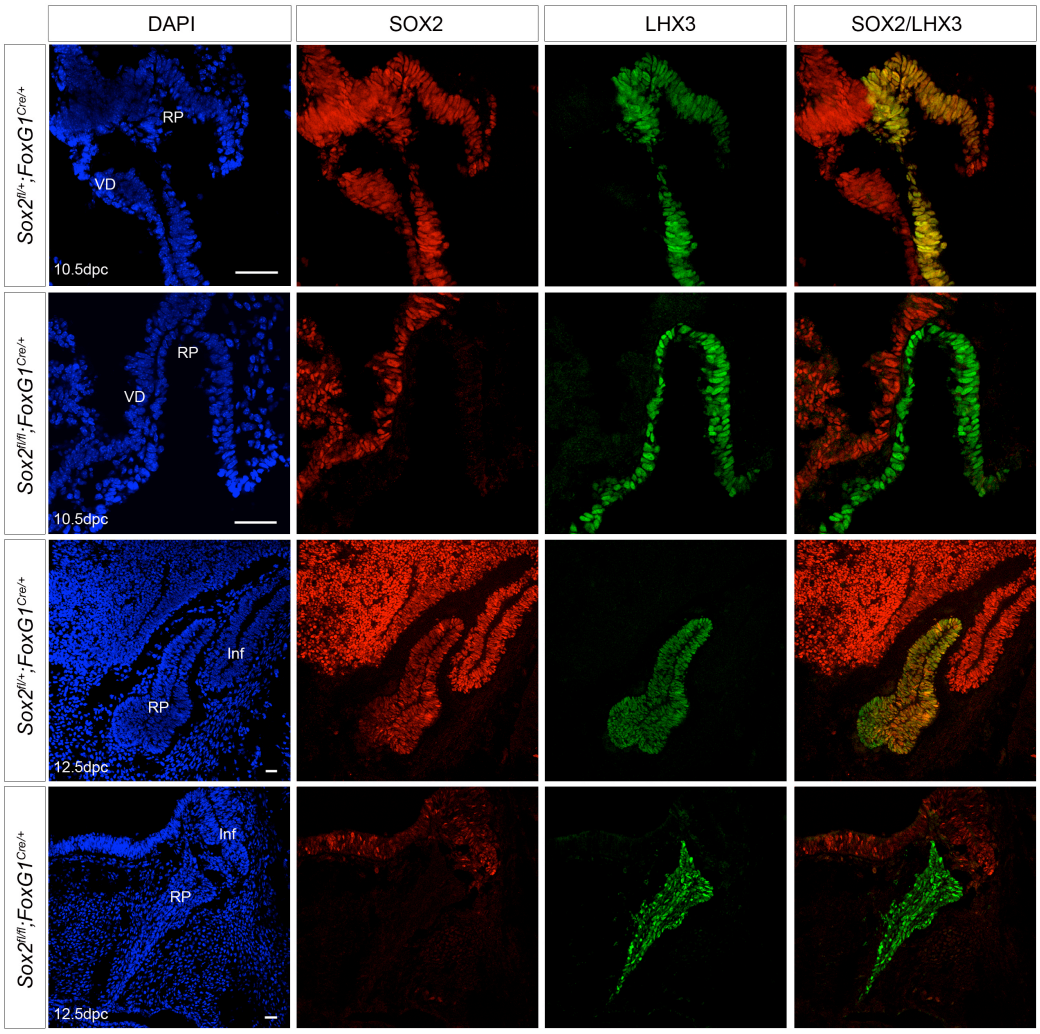


Figure 4.10: LHX3 expression in RP at 10.5dpc and 12.5dpc following *Sox2* deletion using *FoxG1^{Cre}*.

Immunofluorescence for SOX2 (red) and LHX3 (green), counterstained with DAPI (blue) at 10.5dpc and 12.5dpc. SOX2 and LHX3 co-localise (yellow) in every cell of RP at 10.5dpc in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos. SOX2 is completely downregulated in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos at 10.5dpc, but *Lhx3* expression is retained. At 12.5dpc SOX2 is downregulated in the ventral most region of RP in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos. LHX3 is still found in all cells of RP in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos and co-localises with SOX2. In mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos at 12.5dpc SOX2 is downregulated in all cells, however LHX3 persists. All sections are orientated sagittally (see figure 1.3 for a schematic representation). Inf = Infundibulum, VD = Ventral Diencephalon, RP = Rathke's Pouch. Scale = 50µm.

Six6 null mice have a phenotype very similar to the one we have described when deleting *Sox2* with *FoxG1*^{Cre} (Li et al., 2002). *Six6* null mice display a hypoplastic pituitary and reduced progenitor proliferation (Li et al., 2002). In addition to this SOXB1 genes have been shown to directly regulate *Six6* transcription in the hypothalamus (Lee et al., 2012). We therefore decided to look at the expression of SIX6 and its paralogue SIX3 upon *Sox2* deletion. SIX6 is expressed ubiquitously in RP at 10.5dpc along with SOX2 in control embryos (Figure 4.11). Upon *Sox2* deletion using *FoxG1*^{Cre} SIX6 is almost completely downregulated, with one or two cells remaining in the ventral region (Figure 4.11). At 12.5dpc SIX6 and SOX2 still co-localize in control embryos (Figure 4.11). In *Sox2* deleted embryos however, SIX6 expression is completely lost (Figure 4.11). The SIX6 paralogue SIX3 is also expressed in all cells of RP at 12.5dpc in control embryos and in parallel with SIX6 it is also downregulated upon *Sox2* deletion (Figure 4.13). In *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos, *Sox2* is not deleted in a significant number of cells until 12.5dpc and a phenotype is not observed until after this stage (Figure 4.12). In line with this timing of deletion, SIX6 is starting to be downregulated in cells of the dorsal RP 14.5dpc (Figure 4.12). At 18.5dpc in Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$ embryos SIX6 is still absent in the AP, indicating it is not re-expressed by another factor later in development, or that the cell lineage that express SIX6 no longer exists (Figure 4.15). Altogether these results suggest that in RP, as observed in other tissues, SOX2 may regulate *Six6* transcription.

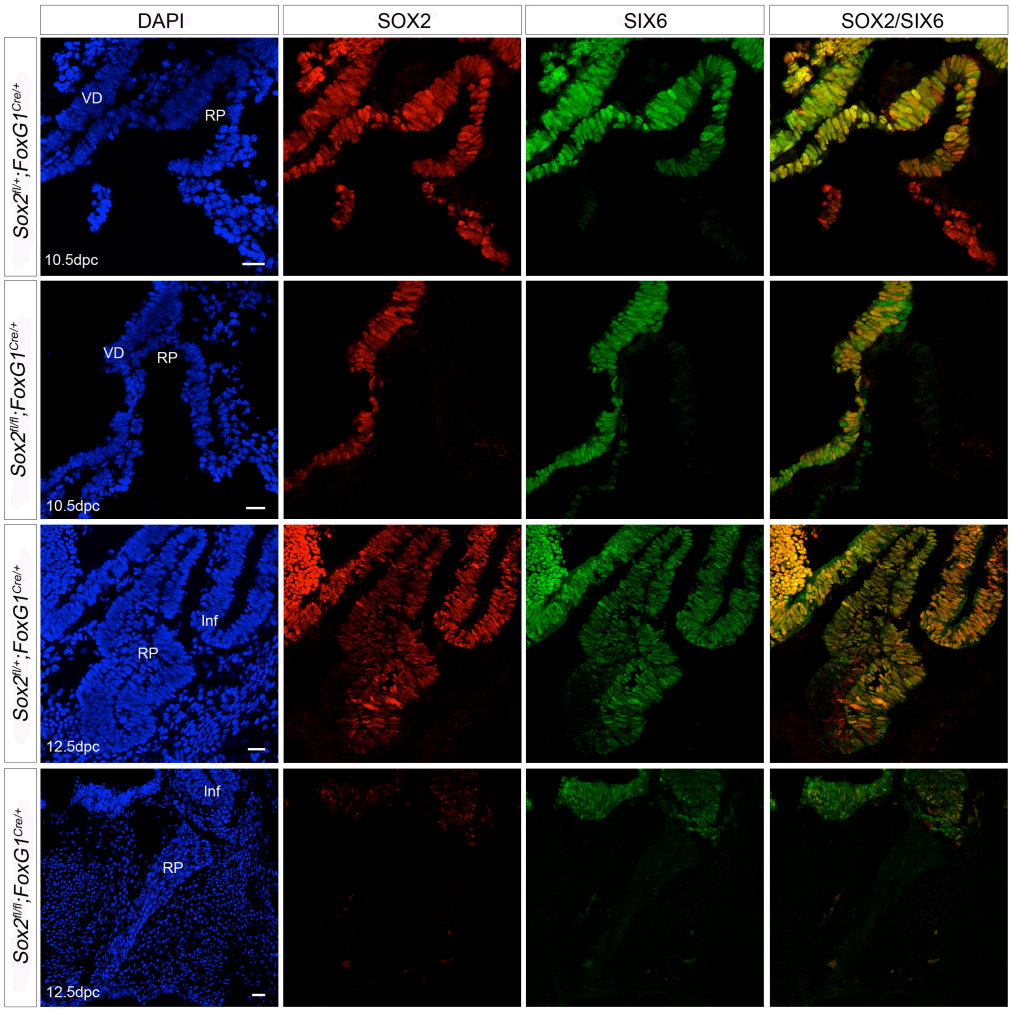


Figure 4.11: SIX6 expression in RP at 10.5dpc and 12.5dpc following *Sox2* deletion using *FoxG1^{Cre}*.

Immunofluorescence for SOX2 (red) and SIX6 (green), counterstained with DAPI (blue) at 10.5dpc and 12.5dpc. SOX2 and SIX6 co-localise (yellow) in all cells of RP at 10.5dpc in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos. In mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos SOX2 and SIX6 are downregulated in all cells of RP, but remain co-localised in the VD. At 12.5dpc in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos SOX2 is lost in the most ventral cells of RP. SIX6 is also lost in the ventral region of RP but co-localises with SOX2 in the rest of RP. Mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos have lost all *Sox2* expression RP and have completely downregulated SIX6. All sections are orientated sagittally (see figure 1.3 for schematic representation). VD = Ventral Diencephalon, RP = Rathke's Pouch, Inf = Infundibulum. Scale = 50μm.

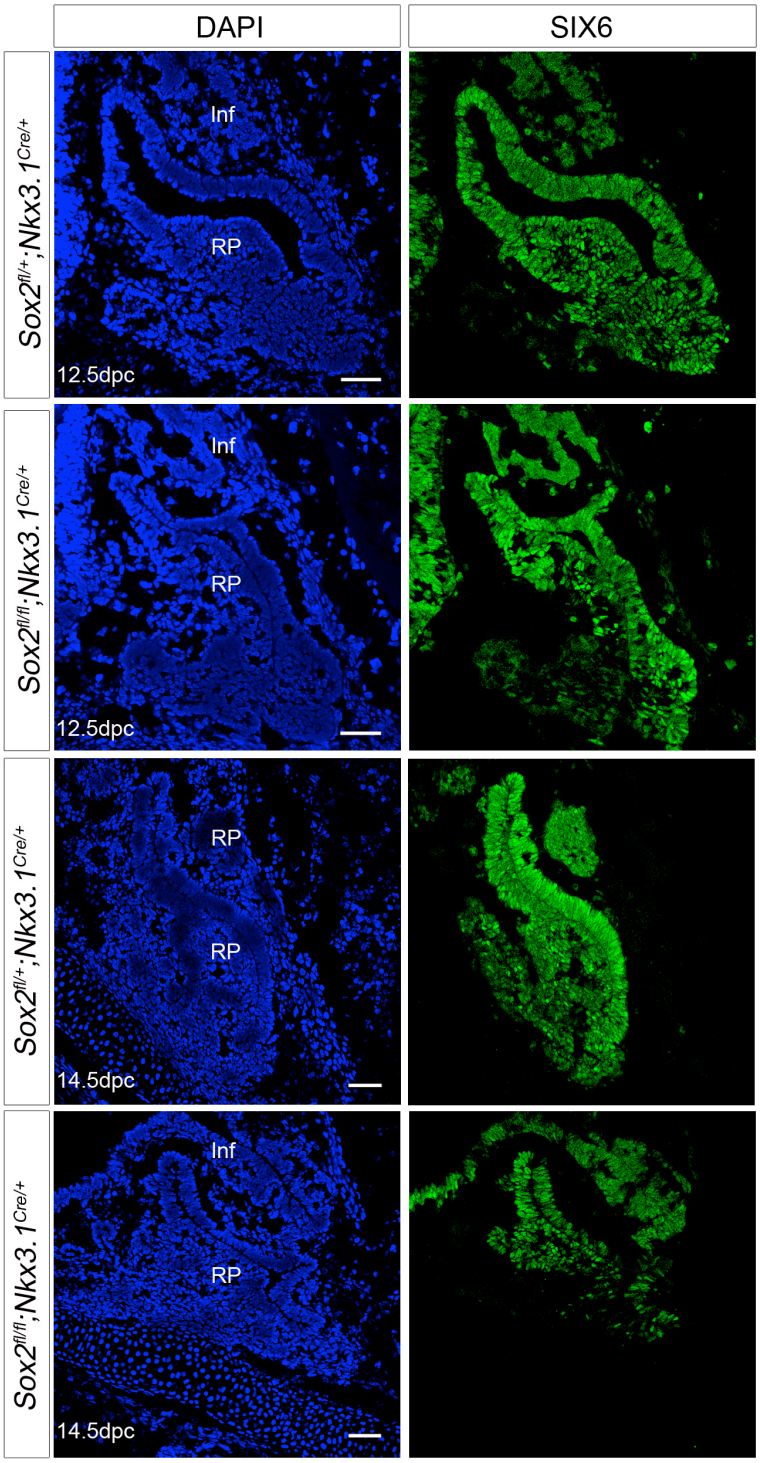


Figure 4.12: SIX6 expression in RP at 12.5dpc and 14.5dpc following *Sox2* deletion using *Nkx3.1*^{Cre}.

Immunofluorescence stain for SIX6 (green), counterstained with DAPI (blue) at 12.5dpc. SIX6 is excluded from the most ventral region of RP in control *Sox2*$^{fl/+}$;*Nkx3.1*$^{Cre/+}$ embryos, becoming progressively dorsally restricted at 14.5dpc. In mutant *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos SIX6 is downregulated in the dorsal region at 14.5dpc. All sections are orientated sagittally. Inf = Infundibulum, RP = Rathke's Pouch. Scale = 50μm.

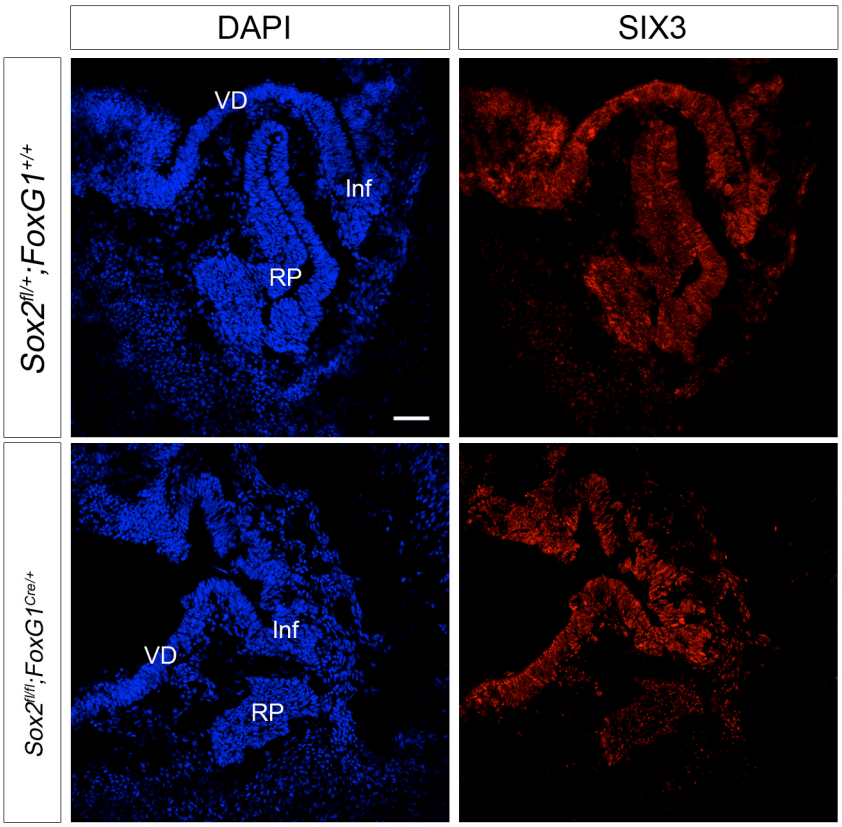


Figure 4.13: SIX3 expression in RP at 12.5dpc following *Sox2* deletion using *FoxG1*^{Cre}. Immunofluorescence for SIX3 (red), counterstained with DAPI (blue) at 12.5dpc. SIX3 is expressed throughout both RP, VD and Infundibulum at 12.5dpc in control *Sox2*$^{fl/+}$;*FoxG1*$^{+/+}$ embryos. At 12.5dpc in mutant *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$ embryos SIX3 expression is strongly downregulated in RP, while expression is maintained in VD and infundibulum. Sections are orientated sagittally. VD = Ventral Diencephalon, Inf = Infundibulum, RP = Rathke's Pouch. Scale = 50µm.

SIX6 expression beyond 12.5dpc has never been described in RP. In addition to this its expression profile in relationship to SOX2 has also never been defined. SOX2 expression in control embryos becomes mainly restricted to the dorsal proliferative zone, around the lumen, as endocrine cells differentiate in the AP (Fauquier et al., 2008). Moreover, we also observe that at 18.5dpc SOX2 expression is maintained in the IL (Figure 4.14). IL melanotrophs are differentiated and express MSH by 16.5dpc (Japon et al., 1994). Levels of expression of SOX2 in IL cells is however weaker than in cells lining the lumen (Figure 4.14). Nevertheless due to the continued expression of SOX2 in the IL, co-localisation is observed with POMC at 18.5dpc in terminally differentiated melanotrophs (Figure 4.16). Like SOX2, SIX6 also becomes dorsally restricted in the AP as development progresses and is also found in IL (Figure 4.14). There is 100% co-localisation between SOX2 and SIX6 in IL at 18.5dpc, however unlike SOX2, SIX6 protein levels remain equally as high throughout the entire IL (Figure 4.14). SOX2 has been previously observed scattered in the parenchyma of the AP (Fauquier et al., 2008), and SIX6 follows this same pattern of expression (Figure 4.14). The co-localisation in the AP between the two proteins differs slightly from the 100% observed in the IL. All $SOX2^{+ve}$ cells are $SIX6^{+ve}$, however not all $SIX6^{+ve}$ cells are $SOX2^{+ve}$ (Figure 4.14). This could indicate either a slightly different role for SIX6 in the AP or the $SIX6^{+ve}$;$SOX2^{-ve}$ cells have just lost SOX2 expression and are in a different phase of the progenitor lineage.

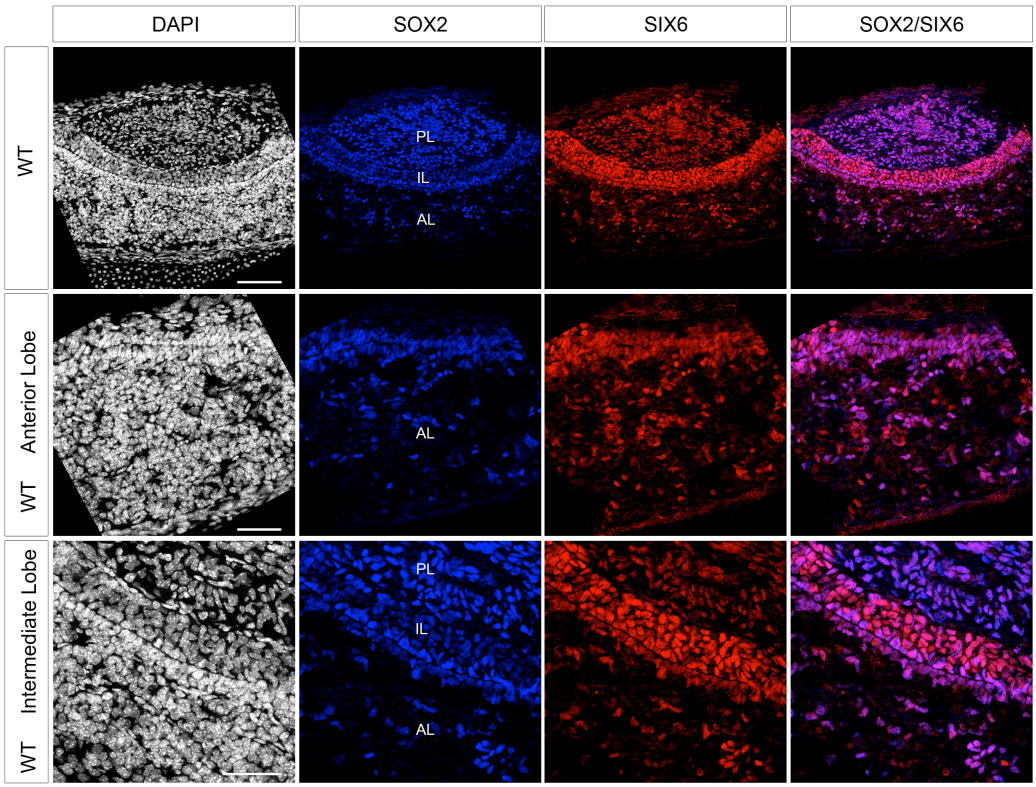


Figure 4.14: SIX6 and SOX2 expression in the anterior and intermediate lobe of WT pituitaries at 18.5dpc

Immunofluorescence for SOX2 (blue) and SIX6 (red), counterstained with DAPI (grey) at 18.5dpc. SOX2 and SIX6 co-localise in the posterior pituitary in addition to the intermediate and anterior lobes. SOX2 and SIX6 co-localise in all cells of the IL, however SOX2 expression is higher in cells that line the lumen, the prospective stem cell niche. SOX2 an SIX6 co-localise in cells that line the lumen in the anterior pituitary and are scattered in the parenchyma. All $SOX2^{+ve}$ cells also express SIX6 in the parenchyma of the anterior pituitary, but not all $SIX6^{+ve}$ cells express SOX2. All sections are orientated coronally. PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 100μm for whole pituitary and 50μm for anterior lobe and intermediate lobe.

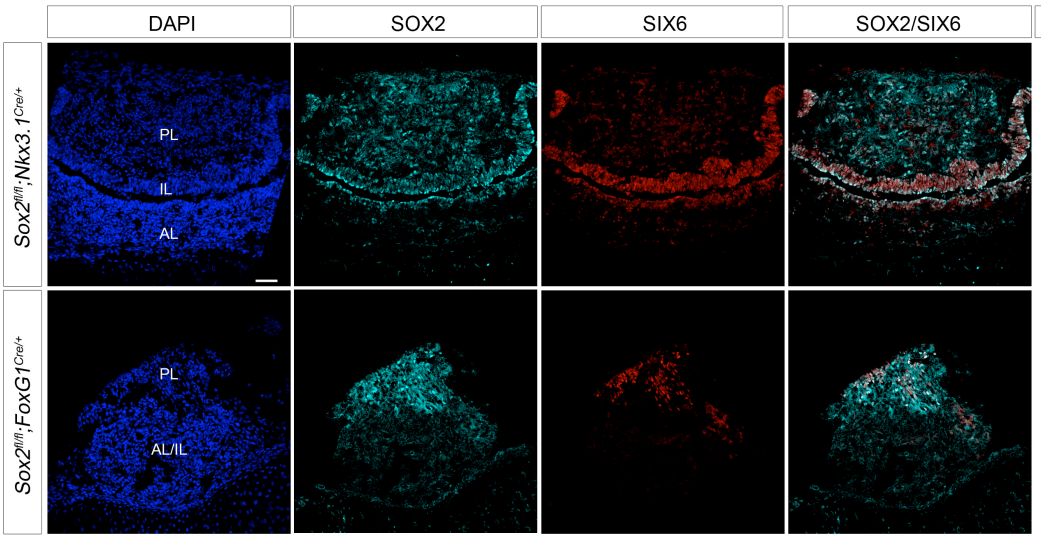


Figure 4.15: SOX2 and SIX6 expression in RP at 18.5dpc following *Sox2* deletion using *FoxG1^{Cre}*.

Immunofluorescence for SOX2 (light blue), SIX6 (red), counterstained with DAPI (blue) at 18.5dpc. SOX2 and SIX6 co-localise in the IL, AL and PL of control *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$*. SOX2 and SIX6 are completely downregulated in all cells of the AP in mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos. Co-localisation is observed in the PL of mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos, however not in all cells. All sections are orientated coronally, control pituitaries represent the midline and mutant pituitaries represent the whole tissue (see figure 1.3 and 1.4 for a schematic representation). PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 50µm.

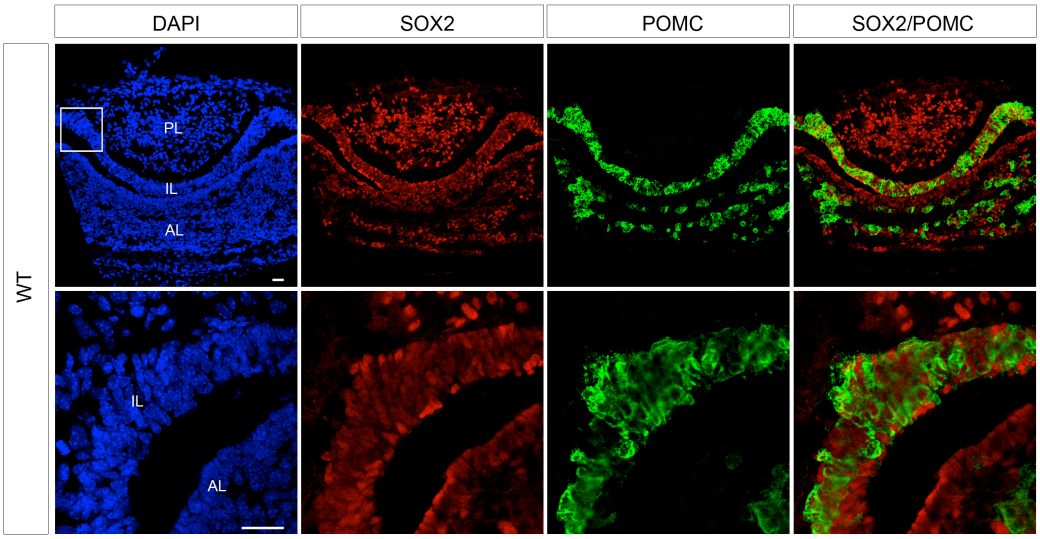


Figure 4.16: SOX2 and POMC co-localisation in the IL of RP.

Immunofluorescence for SOX2 (red), POMC (green), counterstained with DAPI (blue) at 18.5dpc. SOX2 and POMC co-localise in the IL, however higher expressing *Sox2* cells that line the limen of the IL do not co-localise with POMC. Both SOX2 and POMC are found in the AP, however co-localisation does not occur. PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Square represents enlarged area in bottom panels. Sections orientated coronally. Scale = 50μm.

4.2.5 Endocrine cell differentiation analysis following *Sox2* deletion

Based on the *Nkx3.1^{Cre}* fate mapping, we hypothesised that all endocrine cell type would be affected by *Sox2* deletion (Figure 3.3). At 18.5dpc, *Sox2$^{fl/fl}$;Nkx3.1$^{cre/+}$* embryos did indeed reveal that all endocrine cell types of the AP and IL were reduced, although to a different extent (Figure 4.17, 4.18A). The percentage of remaining endocrine cells in each population compared to control matched pituitaries were as follows: GH 19.4% (±5.8, n = 3), TSH 35.2% (±17.2, n = 3) LH 25.8% (±8.9, n = 3), PrL 14.7% (±4.9, n = 3), POMC (ACTH) 45.5% (±24.7, n = 2) and POMC (MSH) 19.7% (±4.1, n = 3) (Figure 4.17, 4.18A). Interestingly the reduction in the number of somatotrophs, thyrotrophs, gonadotrophs and lactotrophs was greater than expected based on the fate mapping (Figure 3.3). This may indicate a non-cell autonomous role for SOX2 in regulating endocrine cell differentiation. Conversely the reduction in the number of melanotrophs was not as large as might be expected. Lineage tracing analysis showed 92.0% of POMC^{+ve} cells were *Nkx3.1$^{cre/+}$;R26R$^{YFP/+}$* at 18.5dpc, indicating around 8% of these cells should retain SOX2 expression following deletion (Figure 3.3B). The discrepancy in the reduction in POMC^{+ve} cells in *Sox2$^{fl/fl}$;Nkx3.1$^{cre/+}$* embryos may be caused by differences in recombination efficiency between *Sox2$^{fl/fl}$* and *R26R$^{YFP/YFP}$* alleles (Liu et al., 2012), allowing some cells to appear YFP^{+ve} without actually having *Sox2* deleted. Conversely there may be compensative proliferation in mutants.

Melanotrophs make up 72.0% (±3.3, n = 3) of cells in the IL at 18.5dpc in control (*Sox2$^{fl/+}$;Nkx3.1$^{cre/+}$*) embryos (Figure 4.18B). If the reduction in the size of the IL was solely down to a reduction in early progenitor proliferation one would expect the proportion of melanotrophs that make up the mutant IL to be the same. However, the proportion decreases significantly in *Sox2$^{fl/fl}$;Nkx3.1$^{cre/+}$* embryos to 23.2% (±3.5, n = 3) (Figure 4.18B). Moreover, many if not all of these melanotrophs are likely to have been generated from progenitors in which *Nkx3.1^{Cre}* was not active. These results therefore suggest a differentiation defect, where the lack of SOX2 in

the progenitors compromises their ability to form melanotrophs (Figure 4.18B).

In *Sox2*$^{fl/fl}$*;FoxG1*$^{Cre/+}$ embryos all endocrine cell types are severely affected at 16.5dpc (Figure 4.19A). The endocrine cell populations that differentiate later: somatotrophs, lactotrophs and gonadotrophs, are more severely affected than thyrotrophs, comprising a sub-population of transient cells, the rostral tip thyrotrophs which are the earliest to differentiate, along with corticotrophs, at 12.5dpc. The effect on corticotrophs appears intermediary between early differentiating thyrotrophs and later somatotrophs. The latter however are still severely reduced in number compared to control *Sox2*$^{fl/fl}$*;FoxG1*$^{+/+}$ embryos (Figure 4.19A). Indicating the loss of SOX2 so early on impairs the differentiation of most endocrine cells.

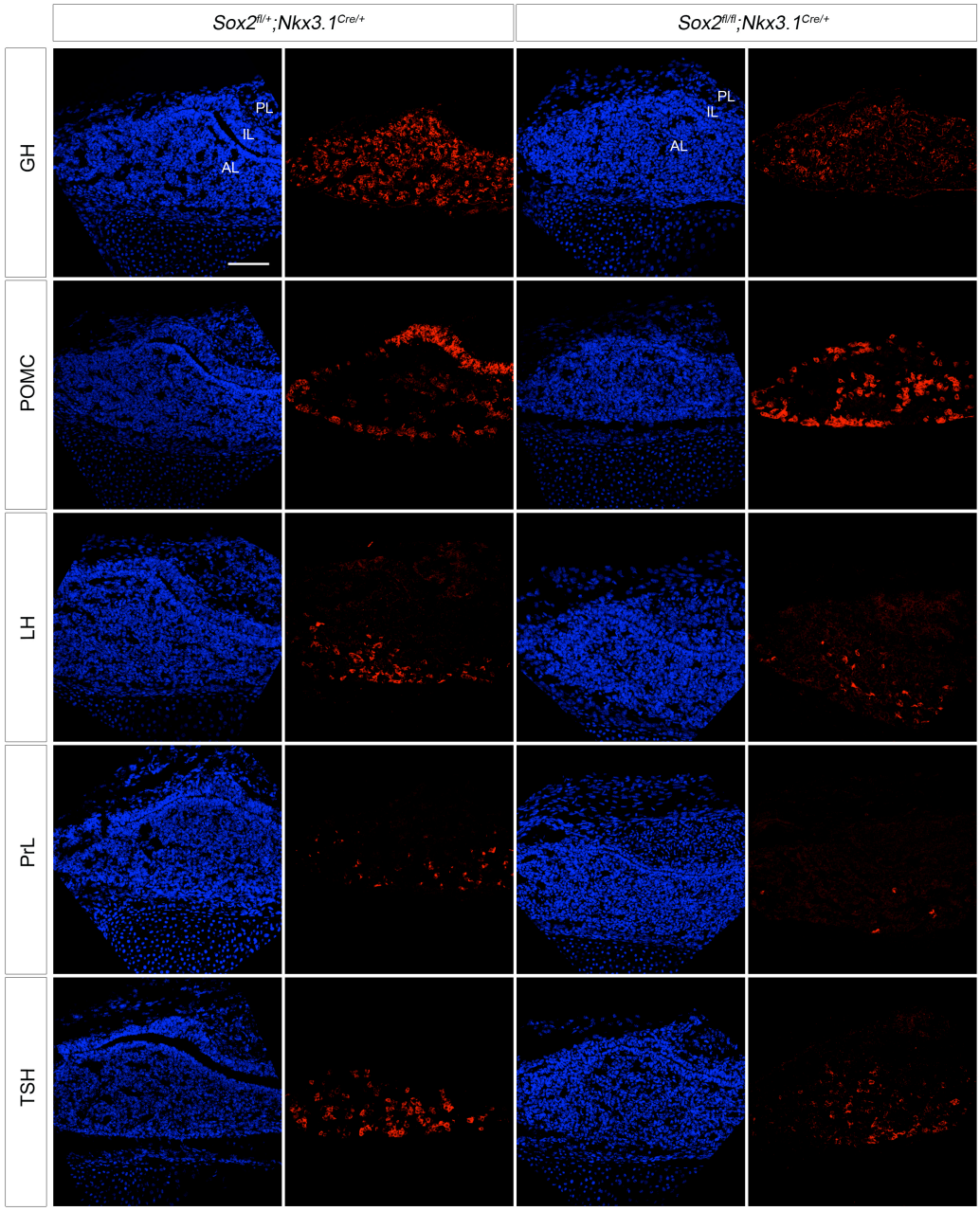


Figure 4.17: Endocrine cell reduction following *Sox2* deletion using *Nkx3.1*^{Cre}.

Immunofluorescence for each anterior lobe endocrine cell type (red), counterstained with DAPI (blue) at 18.5dpc. All endocrine cell populations are reduced, to different extents, in *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos. The AL and IL have become hypoplastic with the IL being more severely disrupted. All sections are orientated coronally and each represents one half of a pituitary (see figure 1.4 for schematic representation). PL = Posterior Lobe, IL = Intermediate lobe, AL = Anterior Lobe. Scale = 100μm.

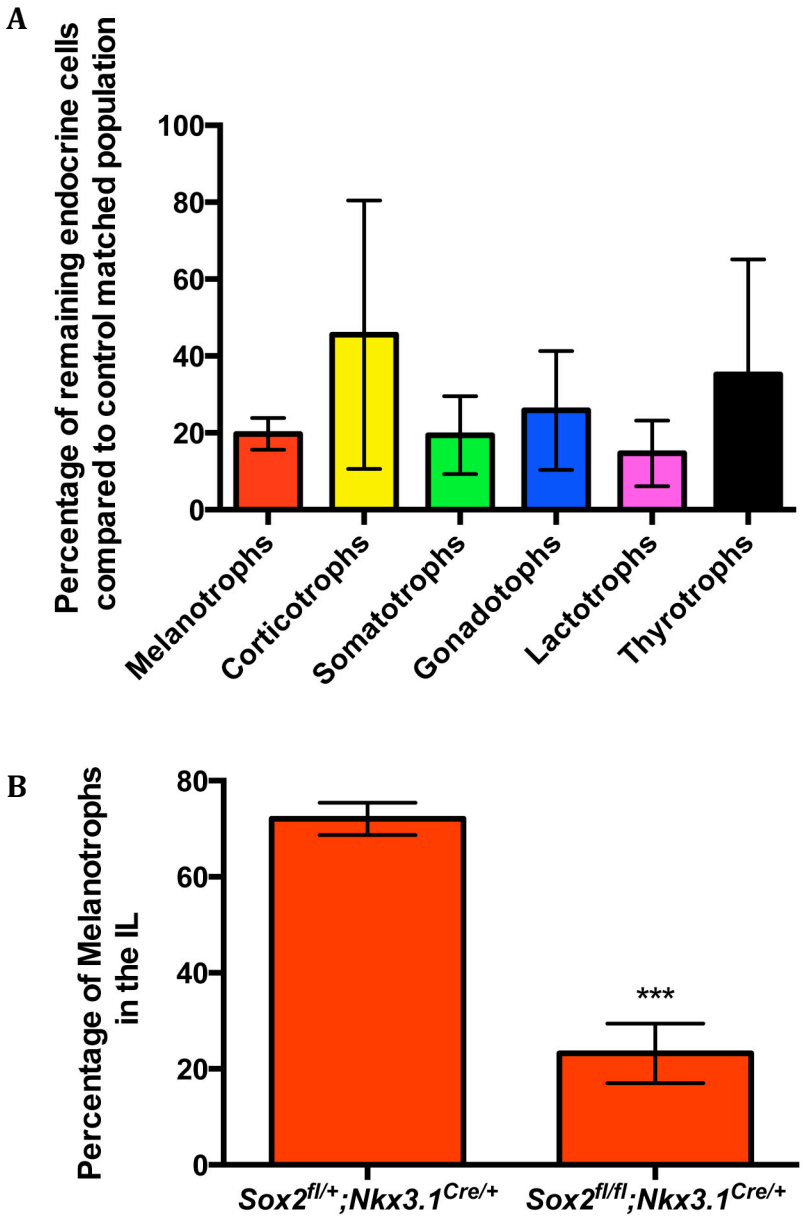


Figure 4.18: Reduction in endocrine cells following *Sox2* deletion using *Nkx3.1^{Cre}*.

A: Percentage of each endocrine cell type remaining in mutant (*Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$)* RP at 18.5dpc compared to the control (*Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$*) matched cell type. Percentages; POMC (MSH) 19.7 (±4.1, n = 3), POMC (ACTH) 45.5 (±24.7, n = 2), GH 19.4 (±5.8, n = 3), LH 25.8 (±8.9, n = 3), PrL 14.7 (±4.9, n = 3) and TSH 35.2 (±17.9, n = 3). **B:** Percentage of melanotrophs remaining in the IL of mutant *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos at 18.5dpc (23.2%, ±3.5, n = 3), is significantly lower than the percentage of melanotrophs in the IL of control *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$* embryos (72.0%, ±3.3, n = 3).

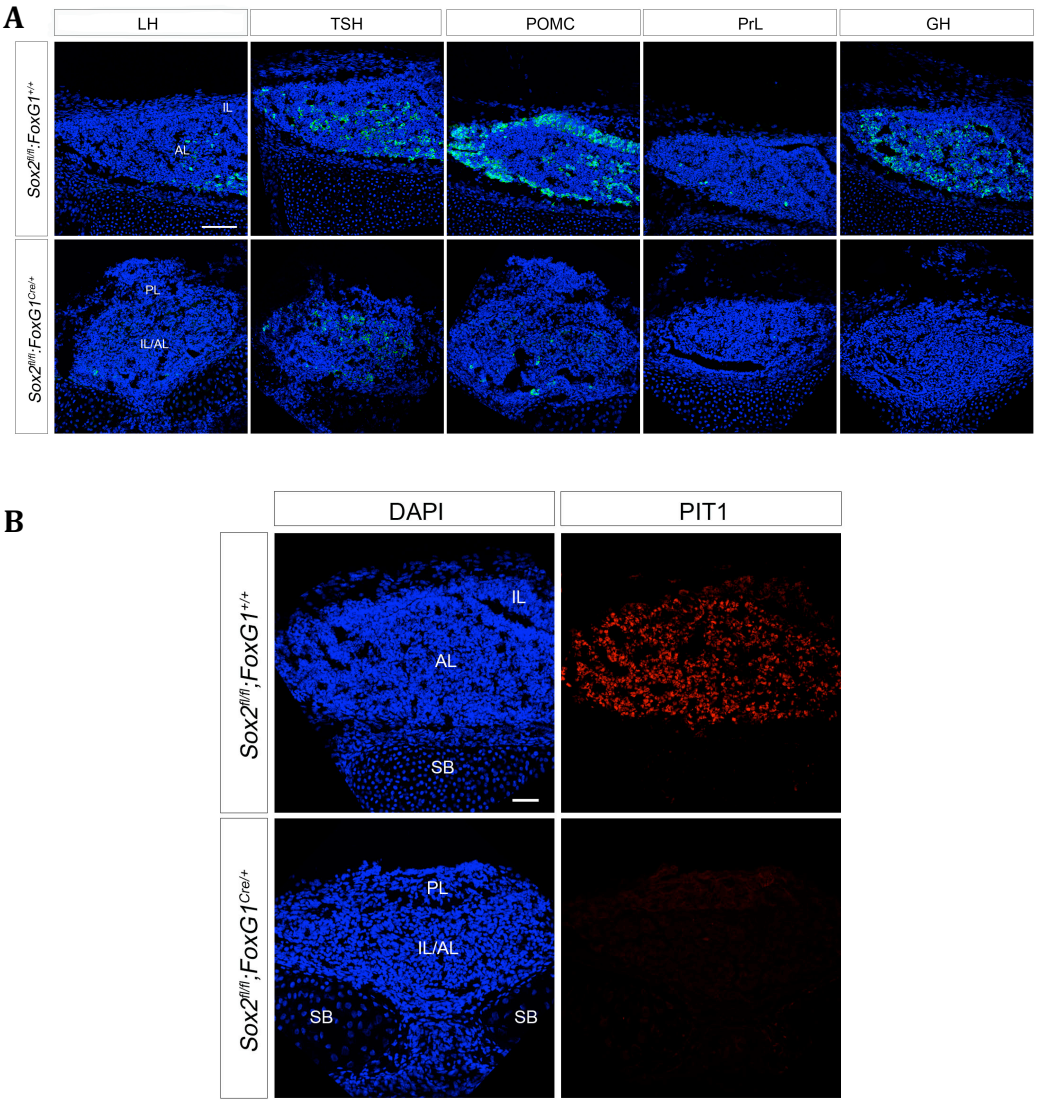


Figure 4.19: Endocrine cell and PIT1 reduction following *Sox2* deletion using *FoxG1^{Cre}*. **A**: Immunofluorescence for each AP endocrine cell type (green), counterstained with DAPI (blue) at 16.5dpc. The gland is hypoplastic and the morphology extremely aberrant with no defined AP and IL in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos. All endocrine cell populations are dramatically reduced in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos. Somatotrophs are completely absent, very few LH positive gonadotrophs and POMC positive corticotrophs and melanotrophs are observed while more TSH positive thyrotrophs persist. All sections are orientated coronally, control represents one half of an 18.5dpc pituitary, mutant represents the whole pituitary at 18.5dpc. PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 100µm. **B**: Immunofluorescence for PIT1 (red), counterstained with DAPI (blue) at 16.5dpc. PIT1 is expressed throughout the AP of the gland at 16.5dpc in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$* embryos. In mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos PIT1 is completely downregulated. All sections are orientated coronally, control represent one half of an 18.5dpc pituitary, mutant represent the whole pituitary at 18.5dpc . PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe, SB = Sphenoid Bone. Scale = 100µm.

Thyrotrophs undergo two waves of differentiation, an early wave at 12.5dpc that represents rostral-tip thyrotrophs and a later wave at 14.5dpc (Davis et al., 2011). In our model the later the endocrine cells differentiate the fewer there are when *Sox2* is deleted. We therefore speculated that the remaining TSH cells are PIT1 independent early differentiating rostral-tip thyrotrophs (Lin et al., 1994). To determine if this is the case we performed immunohistochemistry staining for TSH at 12.5dpc, just after rostral-tip thyrotrophs are known to appear (Davis et al., 2011). In addition we performed immunohistochemistry staining for PIT1, a marker of non-rostral-tip thyrotrophs. In control embryos TSH is expressed at the most rostral side of the ventral RP (Figure 4.20). Upon *Sox2* deletion TSH is still expressed in the most rostral side of the RP remnant, and extends down between the un-fused sphenoid bone (Figure 4.20). Conversely, in the *Sox2* deleted RP at 16.5dpc PIT1 is completely downregulated (Figure 3.19B). These data suggest that the TSH cells that remain at 16.5dpc are rostral-tip thyrotrophs and are the only significant population of endocrine cells that remain. In addition it indicates that only early differentiating endocrine cells survive *Sox2* deletion.

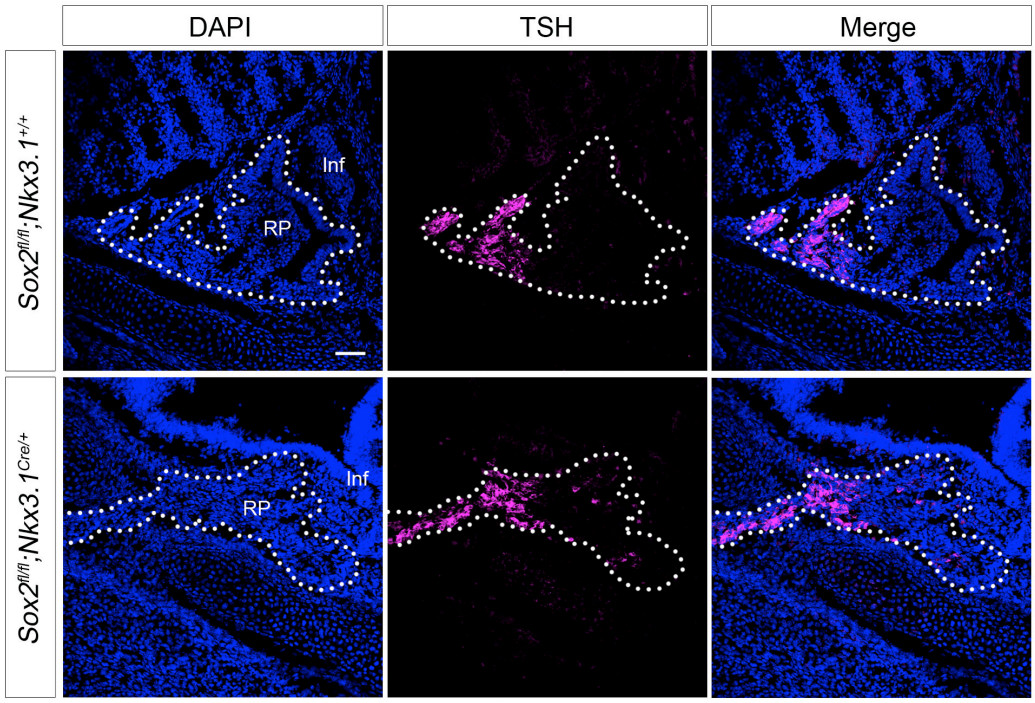


Figure 4.20: Rostral-tip thyrotroph expression following *Sox2* deletion using *FoxG1^{Cre}*. Immunofluorescence for rostral-tip thyrotrophs (pink), counterstained with DAPI (blue) at 14.5dpc. Rostral-tip thyrotrophs are present in the ventral most region of RP at 14.5dpc in control *Sox2$^{fl/+}$;FoxG1$^{+/+}$* and mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos. The sphenoid bone has failed to fuse in mutant *Sox2$^{fl/fl}$;FoxG$^{Cre/+}$* embryos resulting in ventrally displaced RP tissue. All sections are orientated sagittally (see figure 3.1 for a schematic representation). Inf = Infundibulum, RP = Rathke's Pouch. Scale = 100μm.

4.2.6 Gli binding site activity in *Sox2* conditionally deleted embryos using *FoxG1^{Cre}*

Failure of the sphenoid bone to fuse is observed in a number of murine mutations associated with SHH signaling pathway (Balaskas et al., 2012). In light of this and due to the failure to form a fused sphenoid bone in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos we decided to breed these mice with a Gli-binding-site reporter strain (GBS$^{GFP/GFP}$) (Balaskas et al., 2012), resulting in *Sox2$^{f/+}$;FoxG1$^{Cre/+}$;GBS$^{GFP/+}$* embryos. As a downstream mediator of SHH signaling Gli expression and accordingly GBS-GFP provides a direct read out of cells that respond to SHH. Consequently any loss of GFP expression, as a result of *Sox2* deletion will us to identify if SHH signaling has been disrupted.

GFP expression is scattered in the ventral region of RP in control *Sox2$^{fl/+}$;FoxG1$^{Cre/+}$;GBS$^{GFP/+}$* embryos at 14.5dpc where progenitors have begun to differentiate, while it is excluded from the rostral-tip thyrotrophs and the dorsal proliferative area of RP (Figure 4.21). In addition GFP is observed in the rostral and caudal areas of the sphenoid bone as well as the mesenchyme surrounding the rostral-tip thyrotrophs, however it is not seen in the infundibulum (Figure 4.21). In mutant *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$;GBS$^{GFP/+}$* embryos GFP expression is completely lost in RP (Figure 4.21). Moreover GFP expression is significantly downregulated in the unfused sphenoid bone, between which the rostral-tip thyrotrophs have extended. These results suggest that *Sox2* deletion has resulted in a disruption in SHH secretion, potentially from the underlying oral ectoderm.

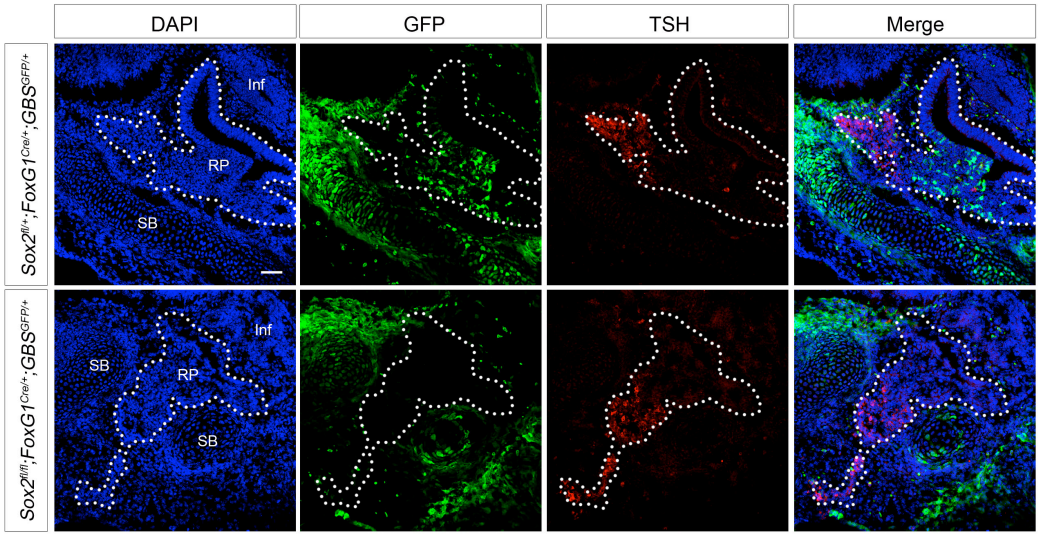


Figure 4.21: TSH expression in RP following *Sox2* deletion using *FoxG1*^{Cre} on a *GBS*^{GFP} background.

Immunofluorescence for GBS^{GFP} (green), TSH (red), counterstained with DAPI (blue) at 14.5dpc. In control *Sox2*$^{fl/+}$;*FoxG1*$^{Cre/+}$;*GBS*$^{GFP/+}$ embryos GFP is expressed in the ventral region of RP where cells are undergoing terminal differentiation, however it is excluded from rostral-tip thyrotrophs (TSH). In addition, GFP is found in the extreeme rostral and caudal areas of the fused spheniod bone. In mutant *Sox2*$^{fl/fl}$;*FoxG1*$^{Cre/+}$;*GBS*$^{GFP/+}$ embryos, GFP is downregulted in the aberrant ventral RP, and remains excluded from the rostral-tip thyrotrophs. Moreover GFP is slightly reduced in the remaining unfused sphenoid bone. RP = Rathke's Pouch, Inf = Infundibulum, SB = Sphenoid Bone. All sections are orientated sagittally (see figure 1.3 for schematic representation). Scale = 50µm.

4.3 Discussion

The experiments carried out in this chapter aimed to investigate the role of SOX2 during pituitary development by analysing the consequences of its conditional deletion on RP progenitors. Two Cre drivers were used to delete *Sox2.* Firstly, *Nkx3.1^{Cre}*, which deleted *Sox2* non-globally and primarily in the dorsal region of RP and secondly *FoxG1^{Cre}*, which globally deleted *Sox2* earlier on in RP. It was hypothesised that the more efficient deletion of *Sox2* using *FoxG1^{Cre}* could result in a more severe phenotype, but that *Sox2* deletion using both Cre drivers would result in a hypoplastic pituitary. Our data showed that this was indeed the case, and that the resulting loss of proliferation may be caused by the downstream loss of SIX6/3 expression.

4.3.1 Pituitary hypoplasia in *Sox2* mutants results from loss of progenitor proliferation and is associated with abnormal expression of cell cycle control proteins

We find that the loss of SOX2 results in a significant reduction of the number of actively proliferating cells in RP when *Sox2* is deleted using either *Nkx3.1^{Cre}* or *FoxG1^{Cre}*, with the reduction being much greater when using the latter. This indicates that the more efficient the deletion of *Sox2* deletion the more pronounced is the reduction in proliferation. A study that was published while these experiments were underway, which used *Hesx1^{Cre}* to delete *Sox2* in RP, had similar findings (Jayakody et al., 2012, see general discussion). Lineage tracing indicated that *FoxG1^{Cre}* would be ubiquitous in RP by 10.5dpc (Figure 3.4). At this stage of development all cells in RP are proliferating and have not begun to exit the cell cycle or to begin differentiation (Davis et al., 2011). *Sox2* deletion throughout the RP at this early stage resulted in its extremely aberrant development. RP pinches off from the underlying oral ectoderm at 11.5dpc (Charles et al., 2005), however in *Sox2$^{fl/fl}$;FoxG1$^{Cre/+}$* embryos this occurs between 12.5dpc-14.5dpc. This 24h+ developmental delay is likely to be a result of the under

proliferation seen in the earlier RP in the early proliferative phase of development prior to cell cycle exit.

The morphological defects in the AP resulting from *Sox2* deletion using *Nkx3.1*^{Cre} are much less severe. This is due in part to the restricted activity of *Nkx3.1*^{Cre}, but also to the timing of its activity. Progenitors for all the different endocrine lineages exit the cell cycle between 11.5dpc and 13.5dpc (Davis et al., 2011). *Nkx3.1*^{Cre} is most active in the AP domain of RP at 12.5dpc indicating *Sox2* deletion may occur after cell cycle exit, when SOX2 is no longer expressed in a subset of the progenitors, thus resulting in a less hypoplastic AP. In the future IL, progenitors exit the cell cycle slightly later, between 12.5dpc-14.5dpc (Davis et al., 2011) hence *Nkx3.1*^{Cre} is active before most progenitors exit the cell cycle as they still express SOX2. Deletion of *Sox2* in this region, results in an IL phenotype as severe as what is seen in the whole of RP when *Sox2* is deleted using *FoxG1*^{Cre}. These two results back up the hypothesis that *Sox2* deletion prior to cell cycle exit results in loss of proliferation and pituitary hypoplasia.

Cyclin D1 regulates the transition between G1/S in the cell cycle (Sala et al., 1992) and is expressed in the dorsal region in RP at 12.5dpc in an area where SOX2 is still expressed (Figure 4.6). SOX2 expression in the developing mammalian cochlea prosensory region (Jacques et al., 2013) and ectopic expression of SOX2 in adult airway epithelial cells in the lung (Tompkins et al., 2011) have both been shown to increase proliferation. Expansion of these cell populations was associated with an increase in Cyclin D1 expression (Jacques et al., 2013; Tompkins et al., 2011). In our model it is unclear whether Cyclin D1 downregulation is a direct result of *Sox2* deletion as seen in previous studies, or if it is a downstream consequence, such as a change in cell fate, and associated with general loss of proliferation. Nevertheless reduced Cyclin D1 expression indicates the loss of proliferation phenotype might be a result of cell cycle arrest and failure to transit through the G1/S checkpoint.

The cell cycle kinase inhibitor p27kip1 is located in the ventral region of RP from E13.5 marking cells that have exited the cell cycle and have differentiated (Bilodeau et al., 2009). Upon *Sox2* deletion using *Nkx3.1*^{Cre}, p27kip1 becomes ectopically expressed in the dorsal region of RP; however in a mutually exclusive pattern from the remaining SOX2^{+ve} cells. Previous studies have indicated that p27kip1 directly participates in SOX2 transcriptional repression in differentiating ES cells (Li et al., 2012). In the adult pituitary of *p27kip1* null mice the number of SOX2^{+ve} cells that line the cleft is increased, and IL tumors develop. Upon deletion of one *Sox2* allele, these two phenotypes are suppressed, highlighting the physiological relevance of this repressive interaction (Li et al., 2012). In contrast, in the early embryonic pituitary, p27kip1 and SOX2 are never co-expressed, demonstrating a different relationship between the two factors at this stage of development (Bilodeau et al., 2009). Our model indicated that the loss of SOX2 causes the ectopic upregulation of p27kip1. This suggests that SOX2 may be repressing p27kip1 expression in proliferating cells, and upon deletion of *Sox2* this repression is lost. In quiescent cells, the relationship between SOX2 and p27kip1 is again different, as it has been shown in the embryonic auditory system that conditional deletion of *Sox2* in post-mitotic supporting cells results in a downregulation of p27kip1 and re-entry in the cell cycle (Liu et al., 2012). In these cells, SOX2 directly activates *p27kip1* expression (Liu et al., 2012). Alternatively, knock down of *Cyclin D1* increases p27kip1 expression through the inhibition of the SKP2 ubiquitin E3 ligase complex (Jonason et al., 2007). This suggests that increased p27kip1 expression may be linked to general loss of proliferation and mediated through Cyclin D1 downregulation. Clearly the relationship between SOX2 and p27kip is complex with each being able to directly regulate each other's transcription, but perhaps with different consequences according to context (Li et al., 2012; Liu et al., 2012).

4.3.2 LHX3 and ISL1 are also required for progenitor maintenance but are unaffected by the loss of SOX2

Analysis of the consequences of genetic deletion of several TFs expressed in early RP indicate they play a role in regulating the proliferation and/or maintenance of progenitors (for review see Kelberman et al., 2006). ISL1 is expressed throughout RP at 9.5dpc before becoming restricted ventrally. Deletion of *Isl1* indicated that the early RP forms but is extremely underdevelopment and fails to develop further than 10.5dpc, possibly indicating it is involved in progenitor proliferation or maintenance (Takuma et al., 1998). In our study, global *Sox2* deletion has no effect on the expression of ISL1 at 10.5dpc, indicating SOX2 does not regulate its expression at this stage.

Sox3 heterozygous mice display a number of pituitary abnormalities including bifurcation during development and hypoplasia in the adult. Due to the lack of SOX3 expression in RP, this is likely a result of perturbed FGF8/10 and BMP4 expression and consequently secretion form the VD (Rizzoti et al., 2004). *Sox2* heterozygous mice also display bifurcation in development and extra clefts in the adult (Kelberman et al., 2006). Furthermore *Sox3* null mice on a *Sox2* heterozygous background have an extremely truncated VD and no infundibulum (Zhao et al., 2011). These studies demonstrate the importance of SOX3 and specifically SOX2 in RP morphogenesis through the correct formation of VD and hence secretion of BMF/FGF. Ectopic $FoxG1^{Cre}$ activity in the VD will have resulted in *Sox2* deletion in a small number of cells in this region, raising the possibility of a disruption to BMP4 and FGF8 expression. Ectopic ventral expression of BMP4 in RP can dorsally expand the region of ISL1 expression, while FGF8 has been shown to inhibit expression in pituitary explants (Treier et al., 1998; Ericson et al., 1998). Following the ventral restriction by BMP4 and FGF8, ISL1 induces the expression of Glycoprotein Hormones α-Subunit (αGSU) and the differentiation of rostral-tip thyrotrophs. These are expanded dorsally upon addition of an FGF antagonist (Ericson et al., 1998;

Norlin et al., 2000). The ventral localisation of PIT1^{-ve}, rostral-tip thyrotrophs at 14.5dpc in our study further suggests ISL1 expression is not disrupted through the ectopic *FoxG1^{Cre}* induced *Sox2* deletion in the VD. This indicates that BMP4 and FGF8 expression and secretion was not disrupted in the VD, providing confidence that the resulting RP hypoplastic phenotype is entirely the result of *Sox2* deletion in RP.

LHX3 is expressed in RP from 9.5dpc and maintained until adulthood (Sheng et al., 1996). Its homozygous deletion has also been shown to result in arrest of RP development primarily due to increased apoptosis (Sheng et al., 1996; Zhao et al., 2006; Ellsworth et al., 2008). SOX2 and LHX3 co-localise in the RP. This is also observed in the sensory epithelium of the developing inner ear (Hume et al., 2007; Rajab et al., 2008). In this system, SOX2 is expressed prior to LHX3 and has been shown to directly activate expression of the *Lhx3* proximal promoter *in vitro* (Rajab et al., 2008). Notwithstanding this, we observe no loss of LHX3 expression in RP upon *Sox2* deletion. This indicates that SOX2 does not control *Lhx3* expression in RP, despite its ability to bind *Lhx3* regulatory regions *in vitro*. *Lhx3* null embryos also fail to express PIT1 and hence fail to produce any somatotrophs, lactotrophs or thyrotrophs (Sheng et al., 1998). In addition *Lhx3* null mice do not form any corticotrophs and gonadotrophs, but do maintain SF1^{+ve} pre-gonadotrophs in an ectopic, more dorsal localisation due to the downregulation of Notch2 (Ellsworth et al., 2008). We also observe a loss of PIT1 expression in addition to a severe reduction in the numbers of gonadotrophs, however in contrast to Sheng et al (1998) this is despite the continued expression of LHX3 at 12.5dpc. These results indicate LHX3 is not responsible for PIT1 down-regulation in our model.

Interestingly ISL1 and LHX3 may regulate progenitor maintenance through the same pathway, at least before 11.5dpc when they co-localise, as ISL1 can bind an 180bp enhancer of *Lhx3* and drive its expression in the pituitary (Mullen et al., 2012). Furthermore, PITX1 may also play a role in this pathway and lie upstream of *Lhx3*, as it has been shown to regulate *Lhx3*

expression in RP (Tremblay et al., 1998; Mullen et al., 2012). Moreover like LHX3 and ISL1, PITX1 is not affected by *Sox2* deletion (Jayakody et al., 2012). This TF network may promote maintenance and proliferation in parallel to SOX2, thus compensating to some extent for the *Sox2* deletion, allowing just sufficient proliferation to form a rudimentary and aberrant pituitary.

4.3.3 *Sox2* deletion causes the downregulation of SIX6/3

A previously published study has shown a role for SIX6 in regulating the proliferation of progenitors in RP. *Six6* null mice have hypoplastic pituitaries and reduced progenitor proliferation (Li et al., 2002). We demonstrate that the conditional deletion of *Sox2* results in an almost exact phenocopy to that seen by Li et al (2002), with reduced progenitor proliferation and pituitary hypoplasia.

SIX6 expression is first observed at 8.0dpc in the diencephalon, from which the hypothalamus in particular will develop (Jean et al., 1999). Throughout development SIX6 is almost always co-expressed with its paralogue SIX3, with both being expressed in the eye, hypothalamus and pituitary (Jean et al., 1999; López-Ríos et al., 1999; Toy & Sundin, 1999). Transcriptional regulation of *Six6/3* in the eye has been extensively described. NeuroD1, LHX2 and PAX6 have all been shown to induce *Six6/3* expression in the retina, where *Six6* overexpression promotes proliferation (Conte et al., 2010; Tétreault et al., 2009). Interestingly despite LHX2 and SIX6 both being expressed in the same region of the VD, *Lhx2* null mice show no change in SIX6 expression. This indicates that control of SIX6 expression is highly tissue specific (Tétreault et al., 2009).

In RP we observe a consistent co-localisation of SOX2 and SIX6 throughout development, up until at least 18.5dp where they co-localise in all three lobes. Moreover the complete loss of SOX2 in mutant mice results in the global downregulation of SIX6 at 18.5dpc. The only other TF known to co-

localise as closely as SIX6 does with SOX2 in RP and pituitary is SOX9 but it does so later, being expressed only from about 14.5dpc (Fauquier et al., 2008; Rizzoti et al., 2013). SOX2 co-localises with SOX9 in 99% of cells in RP, however this is only in the AP, as SOX9 is not found in the IL (Fauquier et al 2008). $SOX2^{+ve}$;$SOX9^{+ve}$ cells in the AP have been shown to be the SC population in RP, capable of forming all endocrine cell types (Rizzoti et al., 2013). In addition both $SOX2^{+ve}$ and $SOX9^{+ve}$ cells can form pituispheres *in vitro* (Rizzoti et al., 2013). It is likely that the $SOX2^{+ve}$;$SIX6^{+ve}$ cells of the AP are also $SOX9^{+ve}$ (after E14.5), indicating $SIX6^{+ve}$ cells may be capable of forming pituispheres in vitro and thus also be a marker for pituitary stem cells. In contrast the $SOX2^{+ve}$;$SIX6^{+ve}$ cells in the IL will not co-express SOX9. This indicates a different identity of these cells in RP. In addition to the absence of SOX9 expression in the IL, weaker SOX2 staining and POMC expression indicates these cells will become terminally differentiated melanotrophs and not SC. SOX2 has been found to co-localise with endocrine cells in the AP, however this is extremely rare and almost always cytoplasmic. The co-localisation of POMC and nuclear SOX2 in the majority of cells in the IL suggests SOX2 may have a different role in these cells compared to the SC of the MC layer of the AP. Furthermore SIX6 expression in all $SOX2^{+ve}$ cells of the IL suggests a large proportion of the $SOX2^{+ve}$;$POMC^{+ve}$ cells will also express SIX6. This indicated that the role of SIX6 in these cells may be different from the proliferative role that has been described for it previously.

Upon deletion of *Sox2* throughout RP with $FoxG1^{Cre}$, SIX6 is completely downregulated and SIX3 expression is also reduced. SIX6 is also downregulated when $Nkx3.1^{Cre}$ is used to delete *Sox2*, although to a lesser extent and only after the loss of SOX2 protein at 12.5dpc. Direct transcriptional regulation of *Six6* and *Six3* by SOXB1 proteins occurs in the ventral forebrain, however this study failed to identify a pituitary specific enhancer of *Six6* (Lee et al., 2012). $Sox3^{-/Y}$;$Sox2^{+/-}$ double mutant embryos display a reduced area of SIX6 expression in the VD (Zhao et al., 2012), although this phenotype may also, at least partially, be a result of reduced

SHH expression, as SOX2 can directly activate *Shh* expression in the VD (Zhao et al., 2012). Our data in addition to the previously published studies indicates SOX2 as a likely direct upstream regulator of *Six6/3* expression.

Developmentally the role of SIX6 and SIX3 appear to be promotion of progenitor proliferation. SIX6 is expanded ventrally in the RP of TCF4 null embryos, and this is correlated with an increase in proliferation (Brinkemeier et al., 2007). Our data shows a downregulation in SIX6 and SIX3 expression following *Sox2* deletion. This is coupled with a reduction in BrdU incorporation, Cyclin D1 expression and ectopic expression of p27kip1. All this suggests that *Six6*/3 are the potential targets of SOX2 that are required to maintain PR progenitor proliferation. Rescue experiments where SIX3/6 expression would be restored in *Sox2* mutants would reveal their relevance in the phenotype. Interestingly Li et al (2002) indicated that SIX6 along with co-repressor Dach2 could bind the 'repressor region' of the *p27kip1* promoter *in vitro*. This provides a possible explanation for the ectopic upregulation of *p27kip1* observed in $Sox2^{fl/fl};Nkx3.1^{cre/+}$ embryos, as the upregulation is only observed where SIX6 is downregulated.

In summary our data indicates a likely relationship between SOX2 and SIX6/3 in regulating RP progenitor proliferation. *Six6/3* appears as ideal candidates for a downstream transcription factor target of SOX2 in RP, a relationship that has been reported to occur also in other tissues. Future experiments to identify if SOX2 can bind *Six6/3* enhancers and drive its expression in RP may reveal if this hypothesis is correct

4.3.4 Early SOX2 expression is required for differentiation of all endocrine cell types

It has previously been shown that all endocrine cells are derived from the $SOX2^{+ve}$ population in the AP (Rizzoti et al., 2013; Andoniadou et al., 2013). We therefore hypothesised that SOX2 expression in RP is similarly necessary for the emergence of endocrine cells. *Sox2* deletion using either

Cre driver resulted in the reduction in each endocrine cell type. Moreover the earlier, global deletion of *Sox2* in RP using *FoxG1^{Cre}* resulted in the loss of nearly all endocrine cell types. One explanation for the loss of differentiation is the severe reduction in proliferation. This may result in the pool of progenitors failing to reach the required size, thus impairing differentiation. This is observed when *FoxG1^{Cre}* is used to delete *Sox2,* where many of the cells remain in a non-endocrine, possibly immature state. Substantiating this hypothesis, the cells that do differentiate are PIT1 independent rostral-tip thyrotrophs, which are one of the earliest to differentiate and exit the cell cycle at 11.5dpc (Lin et al., 1994, Davis et al., 2011). The proliferation that occurs up until 10.5dpc may be enough to allow for their differentiation at 11.5dpc.

In addition to the reduction in proliferation, the maintenance, or loss of TF expression following *Sox2* deletion may also affect endocrine cell differentiation. For example the rostral-tip thyrotroph population can be expanded by an increase in ISL1 and LHX3 expression (Norlin et al., 2000). Global *Sox2* deletion has no effect on the expression on either of these TF, and may allow rostral-tip thyrotroph differentiation to occur normally. In contrast to this, PIT1, which is required for the differentiation of somatotrophs, lactotrophs and thyrotrophs (Simmons et al., 1990), is almost completely downregulated following *Sox2* deletion. Upstream of PIT1, the paired homeodomain TF PROP1 is expressed in RP, along with *Sox2* (Yoshida et al., 2009). *Prop1* null mice fail to express PIT1, resulting in impaired somatotroph, lactotroph and thyrotroph differentiation (Anderson et al., 1995; Yako et al., 2011). This may indicate an indirect affect of SOX2 on endocrine cell differentiation via the regulation of PROP1 expression.

Surprisingly, *Sox2* deletion using *Nkx3.1^{Cre}* resulted in a greater reduction in the differentiation of endocrine cells than was predicted. Fate mapping data predicted that approximately 50% of the future somatotrophs originated from *Nkx3.1^{Cre}* positive progenitors and would therefore be potentially affected by SOX2 loss however the observed reduction was 80%. The reason

for this discrepancy is unclear, however it may be due to non-cell autonomous effects following *Sox2* deletion. This is in addition to the disruption that has occurred to the local RP environment resulting from the loss of proliferation and therefore subsequent lack of cells. Wnt signaling and βcatenin accumulation in RP progenitors actively promotes progenitor proliferation (Gaston-Massuet et al., 2011; Kioussi et al., 2002). Thus any disruption of this pathway or disruption of Wnt signaling in the RP environment may perturb RP proliferation and hence endocrine cell differentiation.

The reduction in the number of melanotrophs in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos was much closer to what was predictions based on fate mapping. Interestingly, however, the proportion of melanotrophs remaining in the IL was considerably smaller than in control embryos. One would predict that the proportion of melanotrophs in the IL would be the same as in control embryos if the phenotype were solely a result of an earlier loss of proliferation. $Nkx3.1^{Cre}$ is not active in the overlying VD/infundibulum, which rules out a non-cell autonomous effect resulting in perturbed endocrine cell differentiation. Consequently a cell-autonomous affect resulting from *Sox2* deletion may be contributing to the loss of melanotroph differentiation in addition to progenitor proliferation. Further analysis of the transcriptional consequence of *Sox2* deletion is presented in chapter 5.

4.3.5 *Sox2* deletion from the oral ectoderm is likely to prevent fusion of the Sphenoid bone

Coupled with pituitary hypoplasia, *Sox2* deletion using $FoxG1^{Cre}$ also results in defects in sphenoid bone fusion. Failure of the sphenoid bone to close is associated with HPE and SOD in humans of which mutations in *SOX2* and *GLI1/2* have been described (Kelberman & Dettani, 2007; Roessler et al., 1996). Similarly we also observe a reduction in GBS^{GFP} expression in the remaining, unfused sphenoid bone. The SHH morphogen binds the Patched (*Ptc1*) receptor, which is often on the membrane of primary cilia (for review

see Quinlan et al., 2008). Interestingly mouse mutations associated with perturbed cilia such as *Polaris*$^{fl/fl}$;*Wnt*^{Cre}, *Ofld1*$^{-/-}$ and *Kif3a*$^{-/-}$ all display an unfused sphenoid bone with RP extending into the resulting cavity. This is in addition to other midline defects (Khonsari et al., 2013). Holoprosencephaly-like defects in mice, in addition to an unfused sphenoid, bone are also associated with homozygous deletion of the SHH agonist Growth Arrest-Specific 1 (*Gas1*) (Martinelli & Fan., 2007). Furthermore, *Gas1* null mice have a severe reduction in *Gli1* expression in RP at 10.5dpc (Khinsari et al., 2013). Because the same phenotype arises from multiple mutations associated with *Shh*, including that described here, it appears likely that correct SHH signaling is required to allow the sphenoid bone to fuse. Previous studies have shown SHH signaling from the VD and oral ectoderm is required for correct RP formation (Treier et al., 1998; Wang et al., 2010). However early *FoxG1*^{Cre} activity in the oral ectoderm (our study and Wang et al., 2010) suggests deletion of *Sox2* in this region that leads to downregulation of GBS-GFP expression and an unfused sphenoid bone, likely due to loss of SHH ventrally. SOX2 directly binds the *Shh* SBE2 enhancer to drive its expression in the VD (Zhao et al., 2012, Trowe et al., 2013). Future experiments will be required to identify if SOX2 can directly activate *Shh* expression in the oral ectoderm.

4.3.6 Conclusions

In conclusion, the studies presented in this chapter investigate the effect of *Sox2* deletion on the development of RP and differentiation of endocrine cells. We show that SOX2 is necessary for the proliferation of RP progenitors. Our data also shows that SIX6/3 are specifically downregulated following *Sox2* deletion, unlike other early expressed TFs. We also indicate *Sox2* deletion disrupts the differentiation of endocrine cells, probably indirectly through the lowering of progenitor proliferation, and the downregulation of the expression of TFs required for differentiation. Finally we indicate the potential loss of SHH signaling in the oral ectoderm prevents sphenoid bone closure.

5. Analysis of Intermediate Lobe proliferation and differentiation defects in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* mutants

5.1 Introduction

In the embryo, the IL develops from the dorsal region of RP, in contact with the infundibulum and separated from the future anterior lobe by the lumen of the pouch. Melanotrophs are the only endocrine cell type generated in the IL and two transcription factors are essential for their differentiation, PAX7 and TPIT (Budry et al., 2012; Pulichino et al., 2003).

The earliest known TF that marks the dorsal region of RP as future IL is PAX7 (Budry et al., 2012). Expressed just prior to TPIT at 15.5dpc, PAX7 exerts its role as a selector gene, selecting for melanotroph identity through chromatin remodeling and opening up new TPIT binding sites in PC2 and DrD2 regions (Budry et al., 2012). TPIT however is a definitive marker of differentiation and essential for POMC expression in melanotrophs and corticotrophs (Lamolet et al., 2001, Pulinchino et al., 2003). An additional important role of TPIT in the IL and possibly the AP is as an inhibitor of the gonadotroph lineage, as *Tpit* null mice ectopically express SF1 in the IL (Pulichino et al., 2003). The IL was the most severely affected region of RP in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos. As a result it was decided to concentrate the subsequent analysis on the phenotype in this area, focusing on the above mentioned TFs.

Melanotrophs are also characterised by their extremely low proliferative index compared to other endocrine cell populations. Moreover in the adult they do not divide at all (Langlais et al., 2013). This quiescence relies on the presence of cell cycle inhibitors, such as p27kip1 as homozygous *p27kip1* deletion results in IL tumors in addition to a more general organ hyperplasia (for review see Quereda & Malumbres., 2009). More specifically, conditional deletion of *p27kip1* in the IL results in hyperplasia, caused by an increase in

melanotroph proliferation (Oesterle et al., 2011). p27kip1 is ectopically upregulated in the dorsal region of RP in *Sox2* mutant embryos and is also associated with a hypoplastic IL (see chapter 4), suggesting a direct or indirect antagonistic affect of SOX2 on p27kip1 expression. We therefore decided to test this hypothesis by removing *p27kip1* on a *Sox2* mutant background, in an attempt to rescue the hypoplastic IL.

5.2 Results

5.2.1 Intermediate lobe cell fate in *Sox2$^{fl/fl}$;Nkx3.1$^{cre/+}$* mutants

The activity of *Nkx3.1^{Cre}* is strongest in the dorsal region of RP (Figure 2.1, 5.1). The consequence of this is a smaller, aberrant IL with fewer POMC^{+ve} (MSH) cells in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* mutants (Figure 3.4 and 3.16). POMC^{+ve} cells are now scattered throughout the IL instead of having a typical pseudo epithelial-like organization (Figure 3.16, 5.1). The resulting thinner IL contains mainly POMC^{-ve} cells (Figure 5.1). To determine their fate, the *R26Reporter^{eYFP}* allele was bred into *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* mice so that the progeny of *Sox2* deleted cells could then be followed.

It has been reported that different floxed alleles are not excised with the same efficiency by Cre recombinase (Liu et al., 2012), therefore expression of eYFP does not necessarily mean that *Sox2* has been deleted. *Nkx3.1^{Cre}* has a robust and lasting activity in dorsal RP (Figure 2.1 and 2.3) and accordingly very efficient deletion of *Sox2* was reproducibly observed in this region (Figure 3.1), so we are confident that, in this case, eYFP expression correlates with *Sox2* deletion.

Lineage tracing revealed that two cell types were produced following *Sox2* deletion in the IL. The first population is POMC^{+ve}/eYFP^{+ve} cells (Figure 5.1). These cells have had *Sox2* deleted by *Nkx3.1^{Cre}* but were still able to differentiate. Secondly, the largest populations of cells were POMC^{-ve};eYFP^{+ve}, and therefore failed to differentiate upon *Sox2* deletion (Figure 5.1).

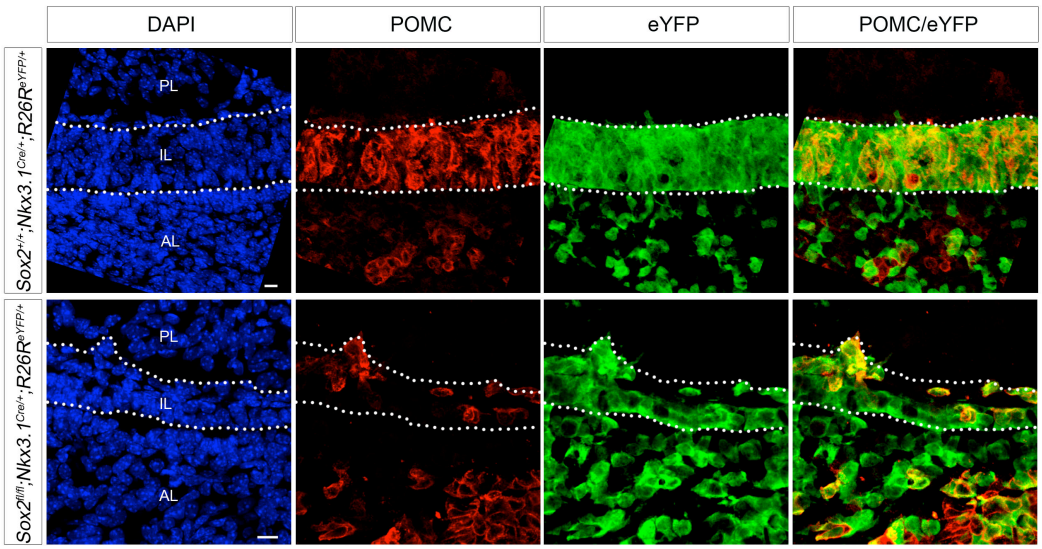


Figure 5.1: POMC expression in *Sox2* deleted cells of the IL and AP of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos.

Immunofluorescence for POMC (red), and eYFP (Green), counterstained with DAPI (blue) at 18.5dpc. POMC (MSH) is expressed throughout the thick pseudo-epithelial IL on control *Sox2$^{+/+}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos and co-localises with eYFP. eYFP expression in the AP of control embryos co-localises with POMC in a small number of cells. POMC expression is reduced in the IL of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos. A few POMC^{+ve} cells in IL express eYFP suggesting that these have differentiated despite likely *Sox2* conditional deletion. Morphologically the IL is only one to two cells thick. POMC is expressed in the AP in eYFP^{+ve} and eYFP^{-ve} cells. These two populations represent cells that have differentiated from NKX3.1^{+ve} cells and are therefore SOX2^{-ve}, and cells that have differentiated from NKX3.1^{-ve} cells and are the progeny of a progenitor that retained SOX2 expression. All sections are orientated coronally. PL = posterior lobe, IL = intermediate lobe, AL = anterior lobe. Scale = 10μm.

Sox2 deletion led to reduced progenitor proliferation and likely cell cycle arrest (Figure 3.5). We then looked at TFs that are expressed in dorsal RP progenitors, prior to their differentiation into melanotrophs, to identify if *Sox2* deleted POMC^{-ve}/eYFP^{+ve} cells had stalled in an earlier pre-endocrine cell state. TPIT is required for terminal differentiation of POMC^{+ve} AP ACTH cells and IL MSH cells (Pulichino et al., 2003). At 14.5dpc in control *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$* embryos, TPIT is expressed in AP corticotrophs (Figure 5.2). It also starts to be expressed in the dorsal region of RP, however in contrast with AP, it co-localizes with SOX2 (Figure 5.2). In 14.5dpc *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, there is a small reduction in the number of TPIT^{+ve} cells in the ventral RP (Figure 5.2). Remarkably, TPIT is not observed in the dorsal region of RP at this stage in mutants (Figure 5.2). By 16.5dpc TPIT is expressed in nearly all cells of the future IL, in addition to AP corticotrophs in *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$* embryos (Figure 5.2). It has also started to be expressed in the future IL of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, however the number of TPIT^{+ve} cells is severely reduced, with many TPIT^{-ve} IL cells (Figure 5.2). Double immunofluorescence for TPIT and POMC in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* embryos showed that TPIT always co-localised with POMC, in a small population of eYFP^{+ve} cells (Figure 5.3). In conclusion, in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, the majority of *Sox2* deleted cells are blocked prior terminal differentiation as they express neither POMC, nor TPIT.

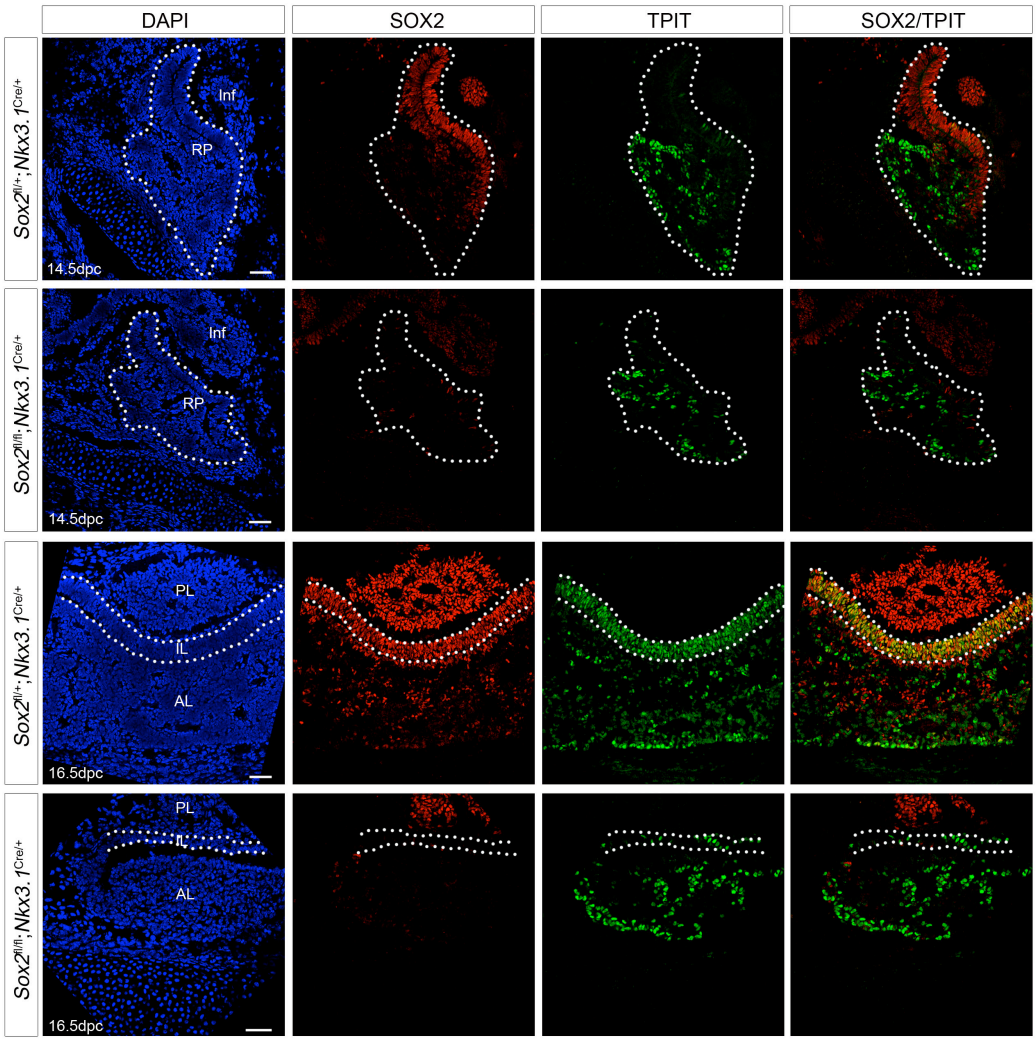


Figure 5.2: TPIT and SOX2 expression in RP following *Sox2* deletion using *Nkx3.1*^{Cre}. Immunofluorescence for SOX2 (red) and TPIT (green), counterstained with DAPI (blue) at 14.5dpc and 16.5dpc. TPIT is expressed in the ventral region of RP and co-localises with SOX2 in a few cells in the dorsal region of control *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ embryos at 14.5dpc. In mutant *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos at 14.5dpc TPIT is downregulated in the ventral region and lost form the dorsal region of RP. By 16.5dpc *Sox2* is expressed throughout the IL and co-localises with TPIT in this region in control *Sox2*$^{fl/+}$*;Nkx3.1*$^{Cre/+}$ embryos while TPIT and SOX2 do not co-localise in the AP. At 16.5dpc in mutant *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos TPIT is slightly downregulated in the AP, however expression in the IL is severely reduced. Sections are orientated sagittally at 14.5dpc and coronally at 16.5dpc (see figure 1.3 and 1.4 for schematic representation). Inf = Infundibulum, RP = Rathke's Pouch, PL = Posterior Lobe, IL = Intermediate Lobe and AL = Anterior Lobe. Scale = 50μm.

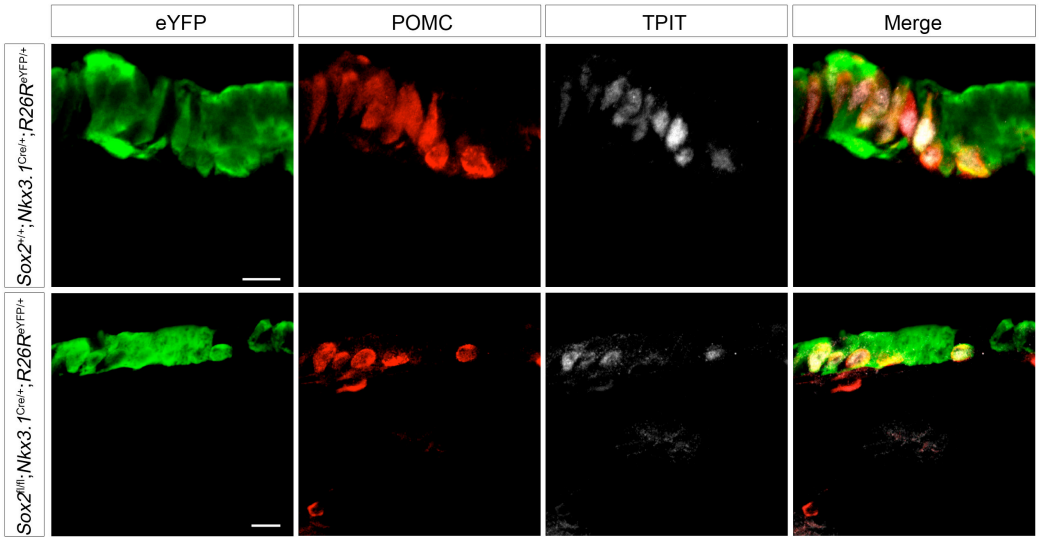


Figure 5.3: TPIT and POMC expression in the IL following *Sox2* deletion using *Nkx3.1^{Cre}*.

Immunofluorescence for POMC (red), TPIT (grey) and eYFP (green), at 16.5dpc. TPIT and POMC co-localise in the IL in control *Sox2$^{fl/+}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* and in the few remaining endocrine cells in the *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$;R26R$^{eYFP/+}$* hypoplastic IL. All sections are orientated coronally. Scale = 10μm.

It has been previously described that cells in the IL of *Tpit* null mice change fate and differentiate into gonadotrophs (Pulinchino et al., 2003). We therefore decided to investigate whether a similar switch of identity occurred in the IL of *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos. SF1 is a TF required for gonadotroph differentiation and is expressed in terminally differentiated LH/FSH cells (Parker & Schimmer., 1997, see chapter 1). SF1 is expressed in the AP of 18.5dpc control *Sox2*$^{fl+}$;*Nkx3.1*$^{+/+}$ and mutant *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos (Figure 5.4). No ectopic SF1 expression is observed in the IL of 18.5dpc *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos indicating that the POMC^{-ve}/eYFP^{+ve} cells have not changed fate and differentiated into gonadotrophs (Figure 5.1).

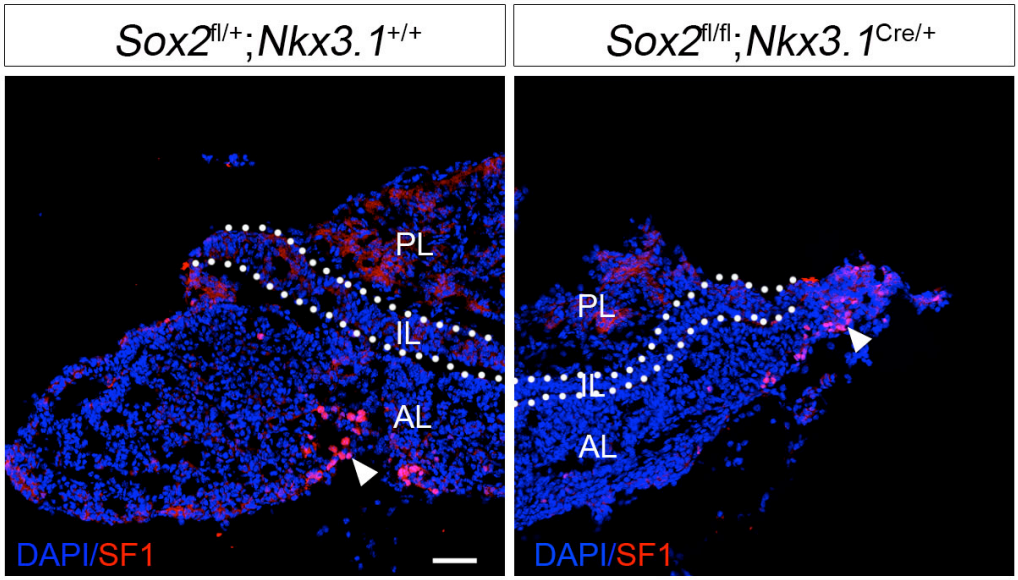
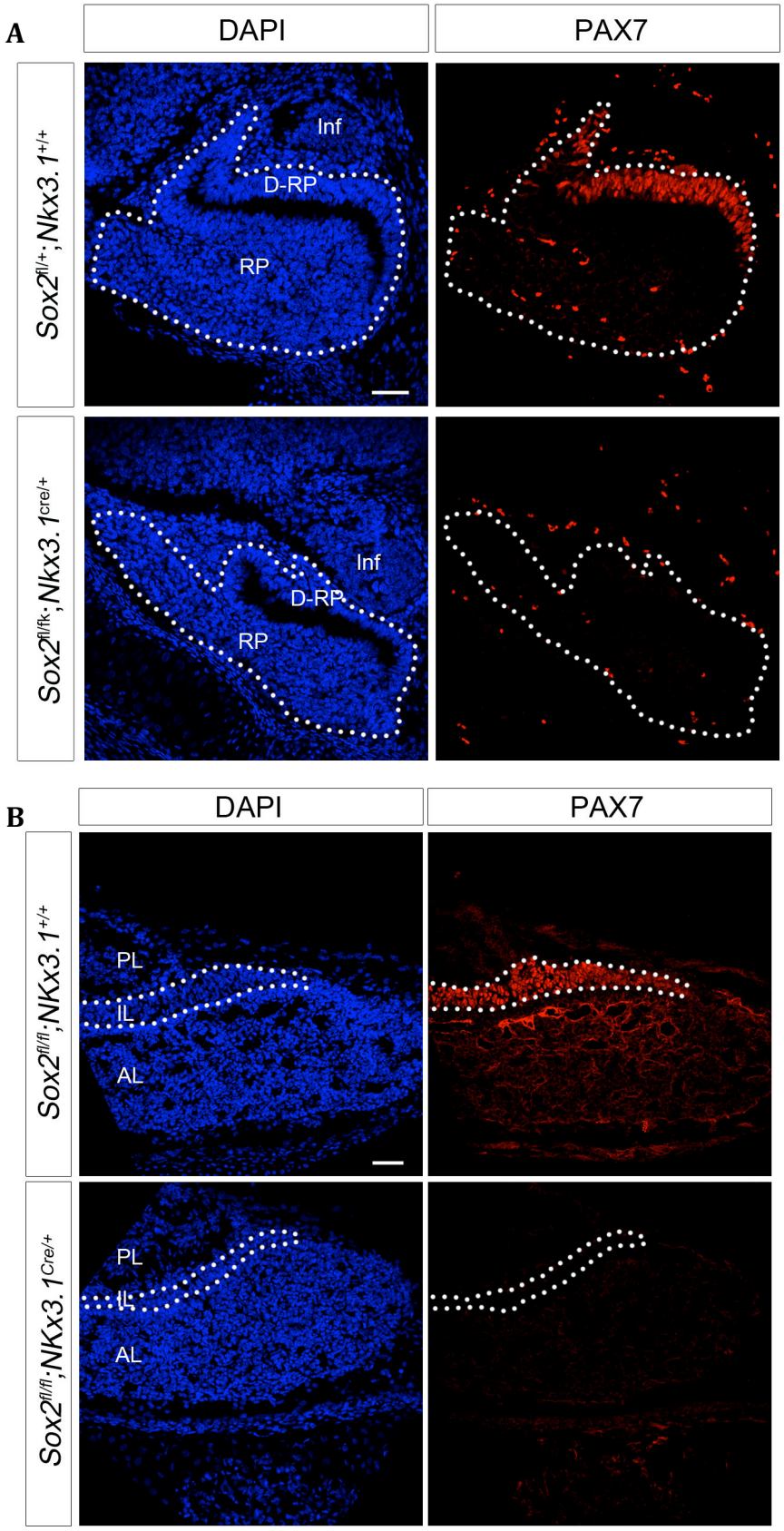


Figure 5.4: SF1 expression in RP following *Sox2* deletion using *Nkx3.1*^{Cre}.

Immunofluorescence for SF1 (red) counterstained with DAPI (blue) at P0. SF1 is expressed in the AP in control *Sox2*$^{fl/+}$;*Nkx3.1*$^{+/+}$ embryos (arrow). High background staining is observed in these sections, primarily in the posterior lobe. Specific gonadotroph SF1 staining is more intense and present, as described (Ingraham et al., 1994) in the ventral region of AL . In the hypoplastic *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ AP, specific SF1 staining is localised more laterally (arrow), however no expression is observed in the IL. All sections are orientated coronally. Scale = 50μm.

The TF PAX7 is expressed in the future IL, earlier than TPIT, and is required for the specification of melanotrophs (Budry et al., 2012). PAX7 appears in the developing IL of control *Sox2*$^{fl/fl}$*;Nkx3.1*$^{+/+}$ embryos at 14.5dpc, restricted to the dorsal region only (D-RP) (Figure 5.5A). By 18.5dpc it is expressed in the future IL, but is excluded from some cells that line the lumen (Figure 5.5C). All POMC^{+ve} cells express PAX7 at 18.5dpc (Figure 5.11). In *Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos, PAX7 is completely downregulated at 14.5dpc and 18.5dpc, in the region where the deletion of *Sox2* is most efficient (Figure 2.1, 5.5A,B). All together these data show *Sox2* deletion blocks proliferation and prevents subsequent differentiation as both POMC and TPIT are down-regulated. It also impairs IL fate acquisition, as the selector PAX7 is absent in mutants. Moreover, as SOX2 and PAX7 co-localise (Budry et al., 2012), SOX2 may be involved in *Pax7* transcriptional regulation.


A
DAPI
PAX7
Sox2fl/+;Nkx3.1+/+
Inf
D-RP
RP
Sox2fl/flk;Nkx3.1cre/+
Inf
D-RP
RP
B
DAPI
PAX7
Sox2fl/fl;NKx3.1+/+
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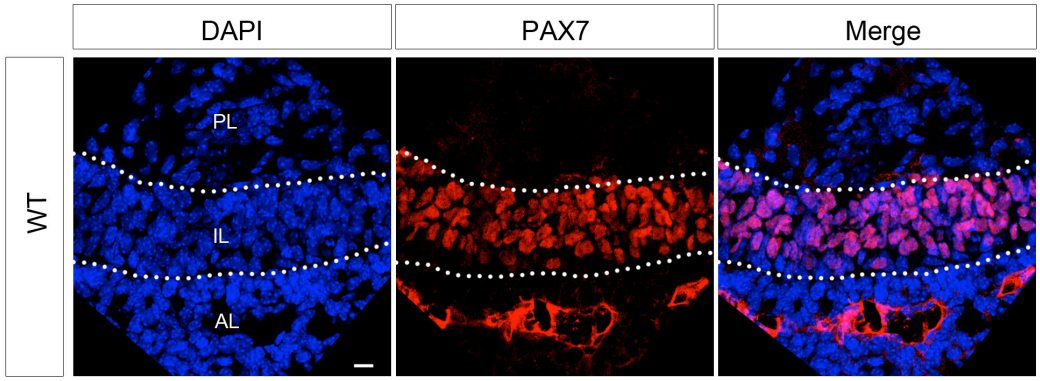

Figure 5.5: PAX7 expression in RP at multiple stages of development.

Immunofluorescence for PAX7 (red) counterstained with DAPI (blue) at 14.5dpc and 18.5dpc. **A:** PAX7 is expressed in the future IL (D-RP) at 14.5dpc in control *Sox2$^{fl/+}$;Nkx3.1$^{+/+}$* embryos. In *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos PAX7 is completely downregulated (remaining red staining is non-specific). D-RP = Dorsal Rathke's Pouch, Inf = Infundibulum, RP = Rathke's Pouch. Sections orientated sagittally (see figure 1.3 for schematic representation). Scale = 50µm. **B:** PAX7 is exclusively expressed in the multi cell layered IL in control *Sox2$^{fl/+}$;Nkx3.1$^{+/+}$* embryos at 18.5dpc, while it is absent in mutant *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos. Sections orientated coronally. Scale = 50µm. **C:** Magnified image of PAX7 expression in the IL of WT (F1 B6;129SvJ) 18.5dpc embryos. PAX7 is expressed in the nuclei of most cells of the IL, however is absent in the cells that line the lumen (interstitial red staining in AL is non-specific). PL = posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. All sections are orientated coronally. Scale = 10µm.

5.2.2 Prospective Melanotroph - Corticotroph fate switch in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* mutants

Dorsal progenitors in *Pax7* null mice retain the capacity to differentiate, however they undergo a change of fate to become corticotrophs, as shown by ectopic glucocorticoid receptor (GR) expression in POMC^{+ve} IL cells (Budry et al., 2012). In control embryos GR is exclusively expressed in AP POMC^{+ve} corticotrophs and is excluded from the IL (Budry et al., 2012) (Figure 5.6). In mutant *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos GR co-localises with all of the few POMC^{+ve} cells present in the aberrant IL at 18.5dpc (arrow) (Figure 5.6). This indicates that the loss of PAX7 in the IL following *Sox2* deletion, likely results in a change of fate from melanotrophs to corticotrophs. The IL of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos contain, therefore, two populations of cells; ectopic corticotrophs (POMC^{+ve}/GR^{+ve}) and non-cycling undifferentiated cells (SOX2^{-ve}/POMC^{-ve}/GR^{-ve}) (Figure 5.6).

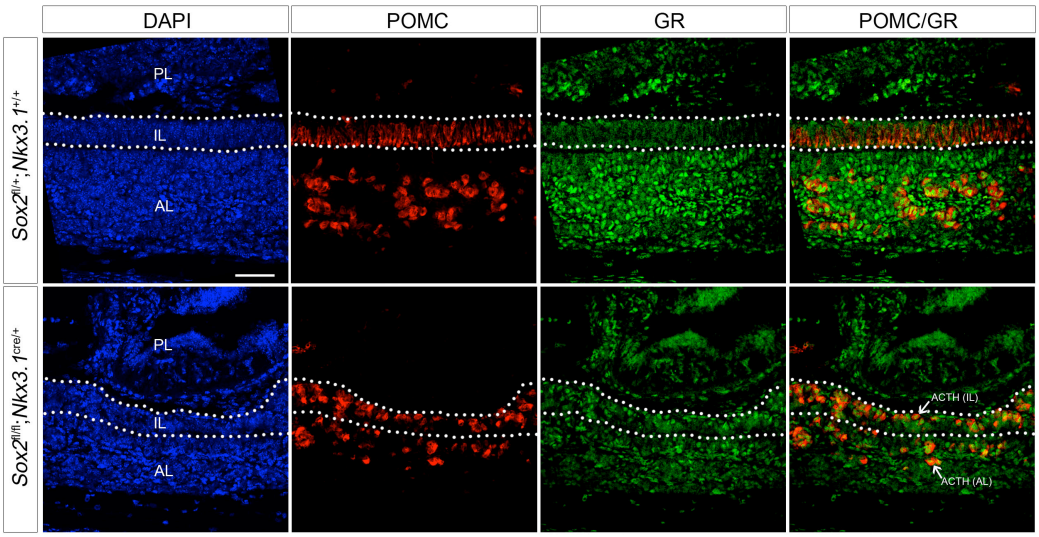

Figure 5.6: POMC and Glucocorticoid receptor (GR) expression in RP following *Sox2* deletion using *Nkx3.1^{Cre}*.

Immunofluorescence for POMC (red), GR (green), counterstained with DAPI (blue) at 18.5dpc. In control *Sox2$^{fl/+}$;Nkx3.1$^{+/+}$* embryos, GR is only exclusively expressed in the AP, in particular in, but not exclusive to, POMC^{+ve} corticotrophs. In mutant *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, GR is ectopically expressed in the IL where it co-localises with POMC, identifying these cells as corticotrophs. All sections are orientated coronally. PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 50µm.

5.2.3 *In Silico* analysis of SOX2 transcription factor binding sites in the *Pax7* genomic locus

The loss of PAX7 in *Sox2*$^{fl/fl}$;*Nkx3.1*$^{Cre/+}$ embryos prompted an examination of the *Pax7* locus for the presence of putative SOX2 binding sites. *Pax7* lies on chromosome 4 in the mouse. Comparative genome analysis with human identified 57 evolutionary conserved regions (ECRs) with a sequence identity ≥ 70% over 100bp in the 66.7kb non-coding region upstream of *Pax7* (www.ecrbrowser.decode.org). Similar analysis of the 76.9kb region downstream of *Pax7* prior to the next gene on chromosome 4, *Tas1r2*, identified 65 ECRs. To lower the number of ECRs identified, the parameters were altered to ≥ 77% sequence identity over 350bp. This restricted the number of total ECRs within the 143.6kb region to 12 (Table 5.1). Thus these highly evolutionarily conserved enhancer regions can be used to identify potential SOX2 transcription TFBS.

Core ECR	Genomic Position	Length	Percentage Identity
1	4:139694780-139695153	374	75.9
2	4:139698566-139699074	509	73.5
3	4:139710556-139711331	776	79.5
4	4:139724767-139725781	1015	81.1
5	4:139736936-139737508	673	75.9
6	4:139832810-139833756	947	76.8
7	4:139839660-139840280	621	79.1
8	4:139841267-139841624	358	76.8
9	4:139852917-139853503	587	76.8
10	4:139878925-139879322	398	78.9
11	4:139879987-139880462	467	78.4
12	4:139905239-139905602	364	76.4

Table 5.1: ECRs upstream and downstream of *Pax7*.

Following identification of the 12 ECRs upstream and downstream of *Pax7*; SOX2 TFBS were searched for, using the www.genomix.de MatInspector software. No hits were detected in the 12 ECRs. In addition to this no SOX2 peaks are observed in these regions in AtT-20 *Sox2* overexpressing cells following Sox2 ChiP-Sequencing (J. Drouin, personal communication). Therefore, it is unlikely that SOX2 directly regulates *Pax7* expression, at least in the 143.6kb we analysed.

5.2.4 Genetic interaction between *Sox2* and *p27* during IL development

5.2.4.1 Intermediate lobe proliferation in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos

Upon *Sox2* deletion using *Nkx3.1^{Cre}*, the morphology and the number of cells of the IL are severely reduced (Figure 5.1). The reduction in proliferation of the progenitors in the IL may be caused by an earlier ectopic upregulation in p27kip1 in the dorsal region of RP (Figure 3.7).

To investigate a possible genetic interaction between *Sox2* and *p27kip1* and potentially rescue the hypoplastic IL, we eliminated both *p27kip1* alleles (Fero et al., 1996) on the *Sox2* mutant background (*Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$*). *p27$^{-/-}$* embryos have not been included in these results and will be analysed later.

We observed an increase in the thickness of the IL in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{cre/-}$* embryos compared to both *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* and *Sox2$^{fl/+}$;Nkx3.1$^{Cre/-}$* embryos at 18.5dpc (Figure 5.7). The increase in the thickness of the IL in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos however, was not uniform as some areas of the IL remained thin similar to the phenotype observed in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos (Figure 5.7). In addition to this the pseudo stratified epithelial-like morphology of the IL was not restored in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos at 18.5dpc (Figure 5.7). The change in IL morphology and subsequent counting performed within its boundary was identified through its structure alone (see materials and methods). This was

due to the loss of the sole IL specific TFs PAX7 and TPIT. Nevertheless the resulting mutant IL was not sufficiently aberrant to prevent the identification of a defined structure, thus providing confidence in the quantification data. Future experiments should aim to identify alternative markers of the IL, to allow for increased accuracy of identification.

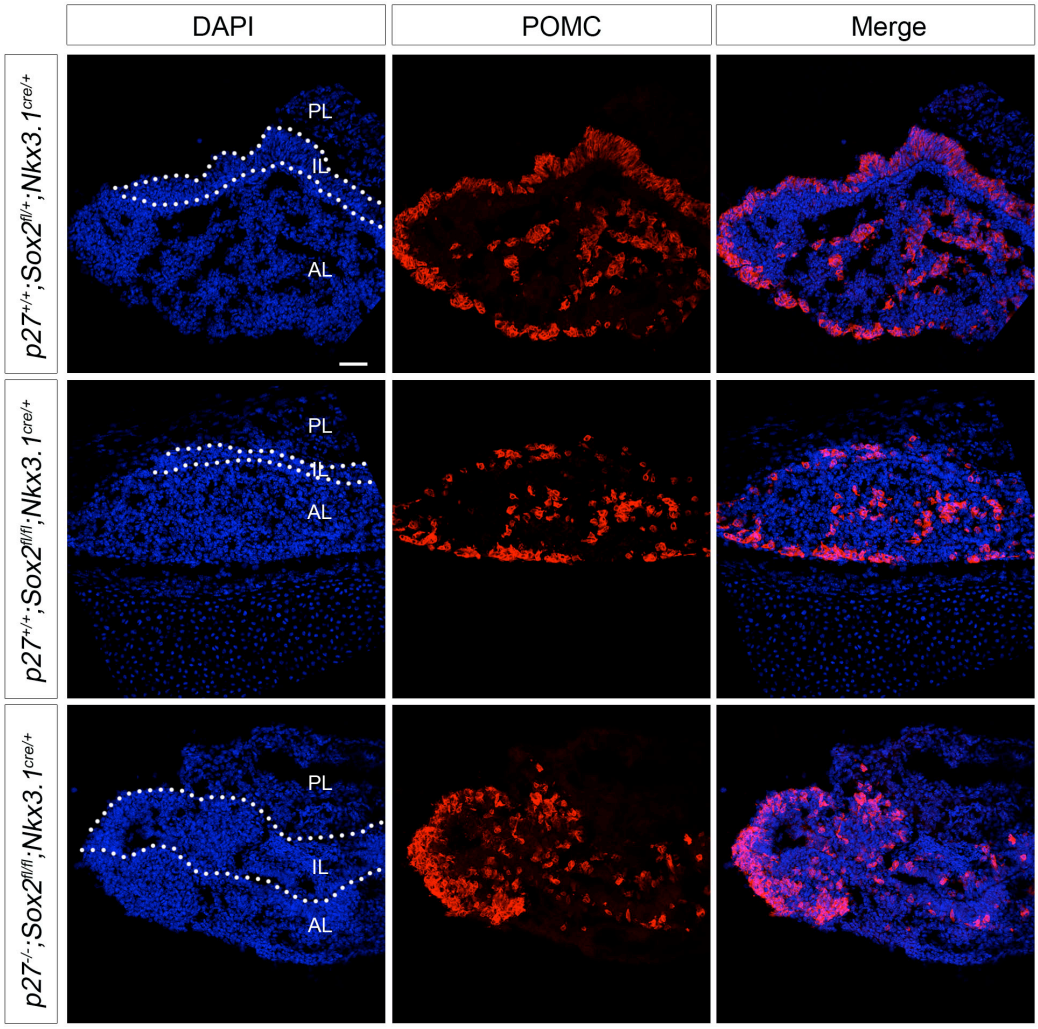

Figure 5.7: Effect of *p27kip1* null mutation on IL size and POMC expression in *Sox2* conditionally deleted embryos.

Immunofluorescence for POMC (red), counterstained with DAPI (blue) at 18.5dpc. POMC is uniformly expressed throughput the IL in control $p27^{+/+};Sox2^{fl/+};Nkx3.1^{Cre/+}$ embryos while it is significantly downregulated in $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos. Removal of both *p27* copies on this mutant background ($p27^{-/-};Sox2^{fl/+};Nkx3.1^{Cre/+}$) results in a significant increase in the size of the IL, however its morphology is still aberrant. POMC expression is also increased compared to $Sox2^{fl/fl};Nkx3.1^{Cre/+}$ embryos, but not as uniform as in control embryos. All sections represent half the pituitary and are orientated coronally. PL = Posterior Lobe, IL = Anterior Lobe, AL = Anterior Lobe. Scale = 100μm.

The number of DAPI^{+ve} nuclei in the IL of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$;p27$^{-/-}$* embryos is significantly increased compared to both control and conditionally deleted embryos (Figure 5.7, 5.8), suggestive of an increase in proliferation. A one-hour EdU pulse was therefore given (via pregnant mothers) at 18.5dpc. The percentage of EdU^{+ve};DAPI cells in the IL in *Sox2* conditionally deleted (*Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$*) embryos was lower (6.9%, ± 1.4, n = 3), than control *Sox2$^{fl/fl}$;Nkx3.1$^{+/+}$* (WT) embryos (10.9%, ± 0.4, n = 3) (Figure 5.9). Upon removal of one *p27kip1* allele of in *Sox2* mutants (*p27$^{+/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+;-}$*) the percentage of EdU^{+ve} cells increased (9.7%, ± 1.2, n = 3) compared to *Sox2* single mutants, but remained below the level observed in control embryos (Figure 5.9). In contrast, the percentage of EdU cells rose above that of control embryos when both *p27kip1* alleles were absent in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos (12.8%, ± 2.7, n = 2) (Figure 5.9). Therefore, dosage of *p27kip1* affects the hypoplastic phenotype of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos and somehow rescues the number of cells in the IL, supporting the existence of a antagonistic interaction between *Sox2* and *p27kip1*. The IL is however morphologically abnormal in these compound mutants.

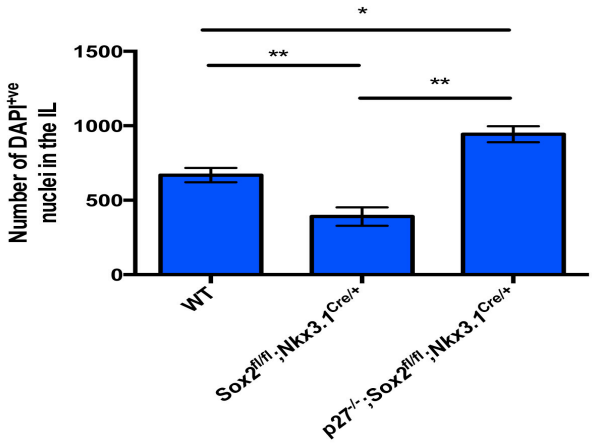

Figure 5.8: Number of DAPI^{+ve} nuclei in the IL of control, *Sox2* conditionally deleted and *Sox2;p27* compound mutants at 18.5dpc.

The total number of DAPI^{+ve} nuclei in *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos (390.3 ±35.7, n = 3) compared to WT (B6;129SvJ) control embryos (668.3 ±48.2, n = 3) is reduced. Loss of *p27kip1* in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos results in a significant increase in the total number of DAPI^{+ve} nuclei (943.0 ±38.0 n = 2) compared to both *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* and control WT embryos.

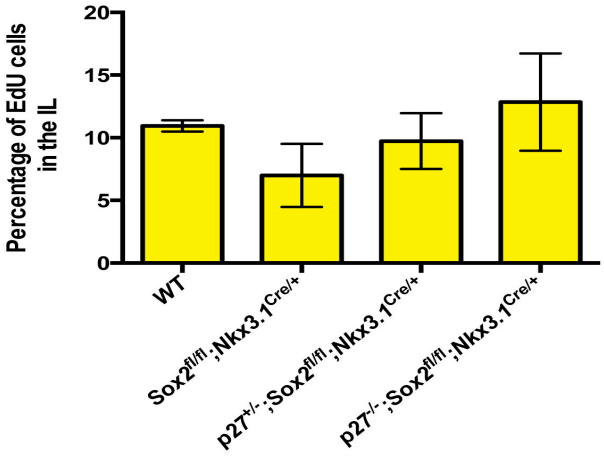

Figure 5.9: Percentage of EdU^{+ve} cells/DAPI in the IL of control, *Sox2* conditionally deleted and *Sox2;p27* compound mutants after 1h00 pulse at 18.5dpc.

There is a decrease in the percentage of cells that incorporate EdU between WT (B6;129SvJ) (10.9% ±0.4, n = 3) and *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos (6.9% ±1.4, n = 3). Reduction of *p27* dosage correlates with further increases in EdU incorporation in *p27$^{-/+}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* (9.7% ±1.3, n = 3) and in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* (12.9% ±2.7, n = 2) embryos.

5.2.4.2 Intermediate lobe differentiation in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos

Conditional deletion of *Sox2* results in a significant reduction in the number of POMC^{+ve} cells remaining in the IL at 18.5dpc (Figure 5.10). The POMC^{+ve} population remaining in the IL represents only 13.3% of the population observed in control embryos. Removal of both *p27kip1* allele in the mutant background induces a large increase in the number of POMC^{+ve} cells, now representing 37.7% of the IL population observed in control matched pituitaries. Despite this increase in the number of POMC^{+ve} cells in the IL of *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, the percentage of POMC^{+ve} cells is still very low at 19.8% (± 0.2, n = 3), comparable to that of single mutants at 14.9% (± 1.7 n = 3) and significantly lower then WT embryos at 72.0% (±3.3, n = 3) (Figure 5.10). This is primarily a result of the highly significant increase in total cell number in the compound mutant IL (Figure 5.8).

We then looked at PAX7 expression. The loss of both *p27kip1* alleles was found to have no effect on PAX7 expression in the IL (Figure 5.11). Similarly in *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos there is no up-regulation of PAX7 expression (Figure 5.11). Therefore, the small increase in the number of POMC^{+ve} cells in the IL of *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos is a result of a substantial increase in the total number of cells in the IL of these embryos, however the cells are still unable to differentiate (Figure 5.10, 5.11).

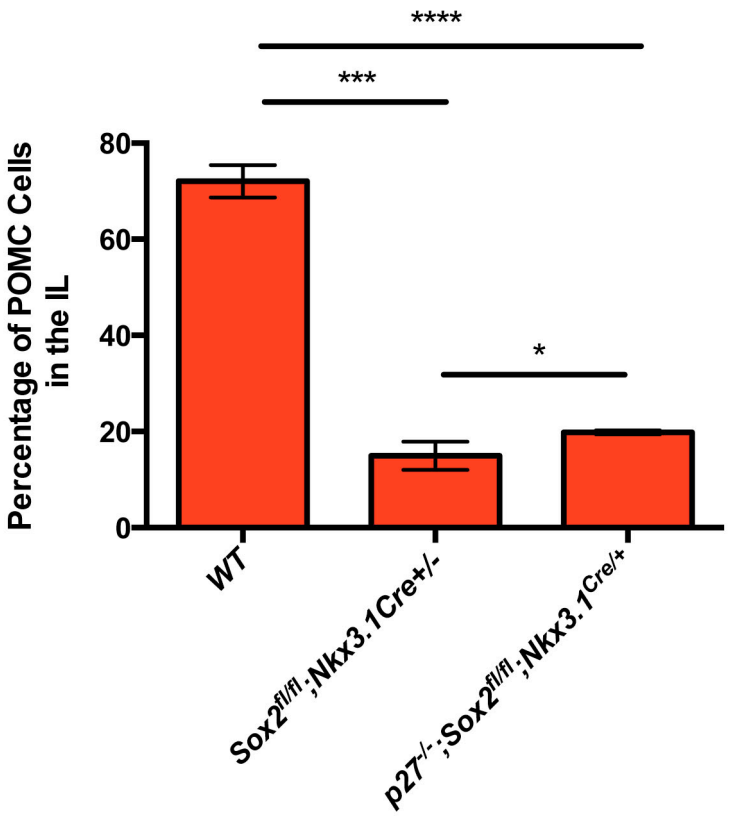

Figure 5.10: Percentage of POMC^{+ve} cells in IL of control, *Sox2* conditionally deleted and *Sox2*;*p27* compound mutants at 18.5dpc.

72.0% (±3.3, n = 3) of cells in the IL are POMC^{+ve} in control WT embryos. This is significantly higher than the percentage of POMC cell in the IL of *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* and *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos displaying respectively 14.4% (±1.6, n = 3) and 19.8% (±0.2, n = 3). There is a slight but significant increase in the percentage of POMC^{+ve} cells in the IL of *p27$^{-/-}$;Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos compared to *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$*.

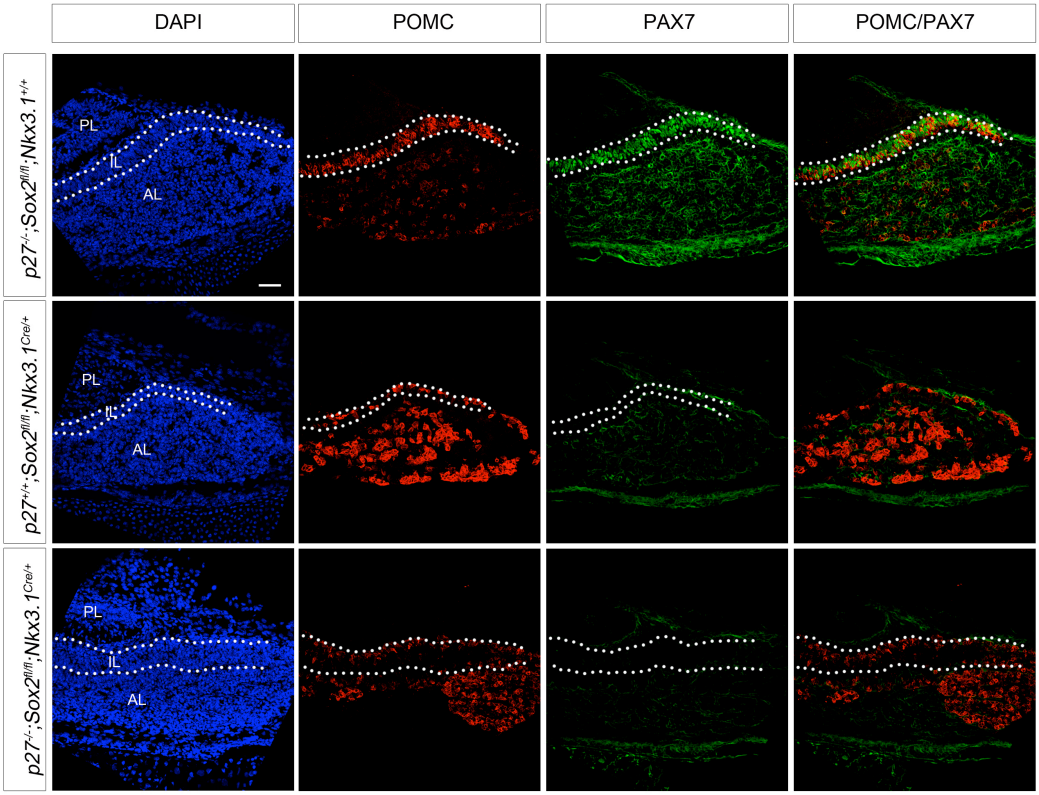

Figure 5.11: PAX7 and POMC expression in *Sox2;p27* compound mutants.

Immunofluorescence for POMC (red) and PAX7 (green) counterstained with DAPI (blue) at 18.5dpc. PAX7 and POMC co-localise in *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{+/+}$ embryos while PAX7 expression is completely lost in both *p27*$^{+/+}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ and *p27*$^{-/-}$*;Sox2*$^{fl/fl}$*;Nkx3.1*$^{Cre/+}$ embryos. Loss of *p27kip1* does not rescue the differentiation phenotype observed in the IL. PAX7 is expressed in the nucleus in the IL; interstitial staining elsewhere is background. All sections represent half the pituitary and are orientated coronally. PL = Posterior Lobe, IL = Intermediate Lobe, AL = Anterior Lobe. Scale = 50μm.

5.3 Discussion

The experiments carried out in this chapter aimed to further investigate the phenotype observed in the IL following *Sox2* deletion using *Nkx3.1*^{Cre}, which is most active in the dorsal region of RP at 12.5dpc, where the future IL will form. Previous results showed a severe reduction in the size of the IL, in addition to endocrine cell numbers. Moreover, a complete downregulation of the IL selector PAX7, inducing a fate change from melanotrophs to corticotrophs in the rare endocrine cells, was found in the mutant IL. Finally, as loss of proliferation was associated with ectopic expression of the negative cell-cycle regulator p27kip1, we attempted to demonstrate an antagonistic genetic interaction and perform a rescue of the mutant phenotype by removing *p27kip1*. Proliferation was restored in these compound mutants but differentiation and cell fate specification was still impaired. These data suggest that SOX2 performs two different roles; early on it is necessary for progenitor proliferation, while subsequently it is required for proper specification, at least in the IL.

5.3.1 *Sox2* deletion in dorsal RP impairs differentiation and may induce a cell fate switch

Fate mapping of *Sox2* conditionally deleted cells revealed the emergence of two populations in IL. The main population is eYFP^{+ve};POMC^{-ve}, and is made of cells that have had *Sox2* deleted and have failed to differentiate, and therefore do not express POMC. The second population, eYFP^{+ve};POMC^{+ve}, represents a minor population that achieved terminal differentiation despite the loss of *Sox2*.

The number of TFs known to be required for the specification and differentiation of dorsal RP progenitors to melanotrophs is relatively small. TPIT is required for the terminal differentiation of both melanotrophs and corticotrophs, with *Tpit* null mice displaying loss POMC expression and a hypoplastic adult IL (Lamolet et al., 2001; Pulichino et al., 2003). TPIT is

normally expressed slightly before POMC in corticotrophs (Lamolet et al., 2001), however it was never observed in eYFP^{+ve};POMC^{-ve} cells. Conversely TPIT was always observed co-expressed with POMC in the eYFP^{+ve};POMC^{+ve} population. This data shows that the eYFP^{+ve};POMC^{-ve} cells have not remained in a TPIT^{+ve} fate, prior to terminal differentiation.

In addition to promoting the differentiation of POMC^{+ve} melanotrophs, TPIT also acts as an inhibitor of the gonadotroph lineage (Pulichino et al., 2003). In the IL of *Tpit* null mice, ectopic gonadotrophs and also PIT1 independent thyrotrophs are present (Pulichino et al., 2003). TPIT is thought to exert its inhibitory function on SF1, a TF required for gonadotroph differentiation, by direct protein-protein interaction, thus sequestering it away from the gonadotroph enhancer (Pulichino et al., 2003). While we observe a dramatic decrease in the number of TPIT^{+ve} cells in the IL, similar to what is observed in *Tpit* null mice, there is no ectopic expression of SF1 in the *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* IL. These results suggest the TPIT^{-ve} (eYFP^{+ve}/POMC^{-ve}) cells are retained in an early progenitor state where they are unable to differentiate into melanotrophs or alternatively, ectopic gonadotrophs.

PAX7 is expressed exclusively in the dorsal region and future IL (Budry et al., 2012; Hosoyama et al., 2010). Unlike in muscle where it has been shown to be required for the maintenance of satellite stem cells (Lepper et al., 2010; Oustanina et al., 2004), PAX7 is thought to act as a selector factor in the developing pituitary, promoting melanotroph identity and differentiation over corticotroph fate (Budry et al., 2012). More precisely, PAX7 opens up condensed chromatin and enhances the activity of TPIT on the regulatory regions of genes specifically expressed in melanotrophs (Budry et al., 2012). In *Pax7* null mice, there is a switch in fate from melanotroph to corticotroph (Budry et al., 2012). This is associated with the ectopic expression of GR in the IL in addition to other corticotroph markers NeuroD1, CRH-R1 and AVP-R1b. In *Sox2* mutants, a complete downregulation of PAX7 is also observed. Furthermore, mirroring the results from Budry et al (2012), we observe a switch in fate from

melanotroph to corticotroph in the eYFP^{+ve};POMC^{+ve} IL population. In both *Pax7* null mice and *Sox2$^{fl/fl}$;Nkx3.1$^{Cre/+}$* embryos, this is indicated by the ectopic expression of GR, normally exclusively expressed in POMC^{+ve} corticotrophs (Budry et al., 2012). Previous experiments have described changes in melanotroph fate due to loss of IL TFs (Pulichino et al., 2003, Budry et al., 2013), however due to the aberrant nature of the mutant IL and AP/IL boundary in our model, migration of POMC^{+ve};GR^{+ve} corticotrophs from the AP to the IL cannot be ruled out. Future experiments will need to be performed to identify if this may account for any of the GR^{+ve} cells observed in the mutant IL. Nevertheless these data suggest SOX2 is specifically required for PAX7 expression and consequently IL identity.

5.3.2 SOX2 and PAX7 may interact to induce IL identity

In contrast with published studies, we consistently observe a low expression level of SOX2 throughout the IL, in addition to the well described high expression in progenitor cells lining the cleft (Fauquier et al., 2008; Himes et al., 2011; Goldberg et al., 2011; Budry et al., 2012). PAX7 is excluded from the cells lining the cleft but in the IL their overlapping patterns of expression strongly suggest that SOX2 and PAX7 co-localise in terminally differentiated melanotrophs. The requirement of SOX2 for PAX7 expression is unlikely to be direct as no SOX2 TFBS were identified on the *Pax7* promoter. This is despite the co-localisation of both proteins that occurs briefly in progenitors when SOX2 is highly expressed (Budry et al., 2012) and later on when SOX2 expression is maintained at a lower level (Figure 4.14, 4.16).

Alternatively, SOX2 and PAX7 might interact. Indeed, in different developmental contexts, the association of SOX and PAX proteins has been extensively described (for review see Kondoh & Kamachi., et al 2010). Most notably, SOX2 and PAX6 interact to activate lens *δ-crystallin* expression. This association does not only promote differentiation, but also initiate lens placode development (Kamachi et al., 2001; Kondoh et al., 2004). It is therefore possible that SOX2 and PAX7 interact to specify some aspects of IL

identity and this interaction may be necessary, directly or indirectly, for PAX7 expression. This is currently under investigation.

5.3.3 *Sox2* and *p27kip1* genetically interact

Previous results indicated an ectopic upregulation of p27kip1 expression in the dorsal region of RP at 14.5dpc. We hypothesised that SOX2, directly or indirectly, represses *p27kip1* expression, therefore resulting in ectopic upregulation in *Sox2* mutants and reduced proliferation. Multiorgan hyperplasia, female sterility, tumorigenesis and predominantly IL tumors are associated with the homozygous deletion of *p27kip1* in mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In addition, deletion of *p27kip1* in the adult cochlea and IL has been shown to increase proliferation (Roussel-Gervais et al., 2010). We therefore removed both *p27kip1* alleles on conditionally deleted *Sox2* mice ($Sox2^{fl/fl};Nkx3.1^{Cre/+}$) to test for a genetic interaction.

While corticotrophs and melanotrophs share a similar transcriptional profile, they differ in their proliferative capacity. Corticotrophs can and do divide, while melanotrophs show a low level of proliferation in the embryo and none in the adult (Langlais et al., 2013). We also observe a low level proliferation in the IL of control embryos at 18.5dpc, which then becomes even lower upon *Sox2* deletion. Strikingly, upon loss of *p27kip1* in the compound mutants, the number of dividing cells in the IL is dramatically increased. In addition, the size of the IL and number of POMC cells are substantially augmented, with the total number of cells in the IL being significantly higher.

The successful restoration of IL proliferation demonstrates that there is a genetic interaction between *Sox2* and *p27kip1* in this region. p27kip1 is initially expressed in the ventral differentiated cells of RP at 13.5dpc (Bilodeau et al., 2009). At this stage SOX2 and p27kip1 expression patterns are mutually exclusive as SOX2 is only found in the dorsal region of RP.

Nevertheless as development progresses p27kip1 expression expands dorsally and becomes ubiquitously present in adults (Bilodeau et al., 2009). It is known that p27kip1 directly represses *Sox2* expression upon ES cell differentiation (Li et al., 2012; see chapter 1). Our results suggest that in turn SOX2 may also be able to repress, directly or indirectly, the expression of p27kip1, so that a negative feedback loop may exist between them as both proteins become co-expressed. SIX6 may be involved, at least in RP, as it was shown to directly repress *p27kip1* (Li et al., 2002) and we show here that *Sox2* deletion induces a complete downregulation of SIX6 (Figure 4.11).

5.3.4 *p27kip1* deletion does not restore differentiation in *Sox2* mutants

Despite the large increase in IL cell number, neither terminal differentiation nor PAX7 expression is restored in compound mutant embryos. This result highlights the un-coupling between proliferation and differentiation, as suggested by Bilodeau et al (2009) where it was shown that sustained proliferation in negative regulators of cell cycle mutants does not affect terminal differentiation in the pituitary.

5.3.5 Conclusions

In conclusion the studies presented in this chapter aimed to further investigate the phenotype observed in the IL following *Sox2* conditional deletion using *Nkx3.1*^{Cre}. Our data shows that PAX7 expression is completely downregulated following *Sox2* deletion and this may result in a change of fate from melanotrophs to corticotrophs. There is likely to be a direct or indirect interaction between SOX2 and PAX7, necessary for IL specification. Moreover, loss of p27kip1 in *Sox2* conditionally deleted mutant's results in restoration of proliferation but still defective differentiation. This is in agreement with the hypothesis of a dual role for SOX2 in RP, initially promoting progenitor proliferation, then being involved in cell type specification, at least in IL. Both roles and also processes appear independent, as previously suggested (Bilodeau et al., 2009).

6. General Discussion

The aim of this thesis was to use genetics to investigate the role of SOX2 during pituitary development. This required conditional, Cre-mediated deletion of the gene specifically in RP *in vivo*. The use of two Cre drivers with distinct but overlapping expression gave both a broader and deeper understanding than would have been possible with either alone. In this chapter I will present a synthesis of these results, propose a model for the role of SOX2 in RP progenitors, discuss still open questions, and suggest directions for further investigation.

6.1 SOX2 is necessary for early progenitor proliferation and, as a consequence, for normal endocrine cell numbers

SOX2 is required for the formation of different organs including the brain (Favaro et al., 2009), eye (Taranova et al., 2006), lung (Que et al., 2009) and ear (Dabdoub et al., 2008; Kiernan et al., 2005), where it is associated in particular with proliferation of progenitors. Previous studies identified SOX2 as a marker for both RP progenitors and adult pituitary SCs (Fauquier et al., 2008; Rizzoti et al., 2013; Andoniadou et al., 2013) (see chapter 1). In light of this we hypothesised that SOX2 may be important in RP progenitors for proper pituitary development.

Our results provide evidence that SOX2 is necessary for RP progenitor proliferation. Performing either global gene deletion thanks to *FoxG1*^{Cre}, or a more restricted one using *Nkx3.1*^{Cre}, we were able to show that SOX2 is required to maintain proliferation in progenitors early in development. This is in agreement with a study published during the course of this work, where the function of SOX2 in RP progenitors was investigated using gene deletion with a different Cre driver, *Hesx1*^{Cre}, and also resulted in severe pituitary hypoplasia (Jayakody et al., 2012). Our two RP Cre drivers also display different temporal patterns of deletion. Deletion of *Sox2* in RP progenitors prior to the main wave of cell cycle exit between 11.5dpc and

13.5dpc (Davis et al., 2011), using *FoxG1^{Cre}*, results in a dramatic loss of proliferation and very severe hypoplasia. *Nkx3.1^{Cre}* activity leads to deletion of *Sox2* in the dorsal region of RP slightly later at 12.5dpc, and while it also results in reduced proliferation, the hypoplasia is not as severe. This allowed a later role for SOX2 to be uncovered, during IL formation (see below).

The severe hypoplasia observed following *Sox2* deletion throughout RP reveals that the protein is necessary for the formation of both anterior and intermediate lobes. This is partially in contrast with the results obtained by Jayakody et al (2012), who also reported obtaining a hypoplastic AP, but a relatively normal IL. This is surprising due to the similarity in the patterns of activity of the Cre drivers used to delete *Sox2*. Both *FoxG1^{Cre}* and *Hesx1^{Cre}* activate *R26R^{eYFP}* in RP by 11.5dpc, prior to the onset of POMC expression in corticotrophs at 12.5dpc (Japon et al., 1994). Using *Hesx1^{Cre}*, complete loss of SOX2 is apparently achieved by 12.5dpc. This is similar to the results obtained here using *Nkx3.1^{Cre}*, in dorsal RP. However, while deletion of *Sox2* using both *Nkx3.1^{Cre}* and *FoxG1^{Cre}* gives rise to a hypoplastic and absent IL, respectively, *Hesx1^{Cre}* does not induce this defect. A possible explanation for this discrepancy may be the *Hesx1* allele itself, in which gene targeting was used to replace the entire *Hesx1* open reading frame with Cre recombinase (Andoniadou et al., 2007). HESX1 is a negative regulator of proliferation in RP (Dattani et al., 1998, Dasen et al., 2001). Therefore, loss of one allele may slightly enhance proliferation, partly counteracting the *Sox2* mutant phenotype. In conclusion, as in several other developing organs, SOX2 is required in RP for progenitor proliferation, and consequently normal pituitary development.

6.2 SOX2, SIX6 and p27kip1 regulate early pituitary progenitor proliferation

6.2.1 SOX2 may directly regulate *Six6* expression

SOX2 and SIX6/3 are co-expressed in RP progenitors. Upon *Sox2* deletion, SIX6/3 are specifically downregulated while other markers of RP progenitors, like LHX3 and PITX-1, are maintained (Fig 6.1) (Jayakody et al., 2012). SOX2 may directly regulate SIX6/3 expression in RP, as it has been shown to do so in different developmental contexts, notably in the CNS (Baccari et al., 2012, Lee et al., 2012, Lee et al., 2013). More precisely, 12 highly evolutionarily conserved regions (ECRs) have been characterized in the *Six6* locus. Two of these recapitulate SIX6 expression in the mouse forebrain (Lee et al., 2012). SOXB1 genes can bind directly to these two enhancers and drive their expression *in vitro*. Conversely *in vivo*, upon *Soxb1* gene dosage reduction, endogenous *SIX6* expression is lost suggesting a direct regulatory action of SOXB1 proteins (Lee et al., 2012). Unfortunately neither of these two ECRs displays activity in RP (Lee et al., 2012). More extensive analysis of the expression pattern induced by the ten remaining ECRs is required. In addition, RP/pituitary SOX2 ChiP-Sequencing would reveal relevant SOX2 TFBS in the *Six6* locus.

6.2.2 *Sox2* and *p27kip1* genetically interact

Upon deletion of *Sox2* using *Nkx3.1*^{Cre}, we observed an ectopic upregulation of CDKI p27kip1 in the dorsal region of RP, where proliferation is reduced. A similar result was also observed by Jayakody et al (2012). We decided to investigate this further by deleting *p27kip1* in *Sox2* mutant embryos. We observed a restoration of dorsal proliferation in compound *p27kip1*;*Sox2* mutant embryos. This demonstrates a genetic interaction between SOX2 and p27kip1 and suggests that SOX2 may repress *p27kip1* expression, at least in the dorsal region of RP. The interaction clearly appears context-dependent. *Sox2* deletion in post-natal cochlear inner pillar cells induces a

downregulation of p27kip1 expression, resulting in proliferation of these normally quiescent differentiated supporting cells (Liu et al., 2012). In this context, the interaction is likely to be direct as SOX2 can bind the *p27kip1* promoter to drive its expression *in vitro* (Liu et al., 2012). In ES cells, and presumably in adult pituitary cells, it is known that p27kip1 recruits a repressor complex to down-regulate *Sox2* expression (Li et al., 2012). However, we have not studied the molecular mechanism, which may be reciprocal, underlying the *Sox2*/*p27* interaction that we have observed in the developing pituitary.

Ectopic p27kip1 expression in the dorsal RP of *Sox2* mutants is also correlated with down-regulation of SIX6. The role of SIX6 to promote cell proliferation has been described, primarily in the developing eye, in various species (Anderson et al., 2012). Overexpression of XOptx2, the *Six6* Xenopous homologue, results in increased expression of Cyclin D1 and a dramatically enlarged eye field (Zuber et al., 1999). Similarly, in Medaka retina, overexpression of SIX6 is associated with increased Cyclin D1 expression and progenitor proliferation (Conte et al., 2010). Interestingly, reduced *p27Xic1* expression is also observed in the SIX6 overexpression retina (Conte et al., 2010; Gestri et al., 2005). Pituitary hypoplasia is present in *Six6* null mice, alongside a reduction in the size of the eye (Li et al., 2002). Significantly, the latter was associated with an increase in p27kip1 expression, while *in vitro* assays demonstrated that SIX6 can directly bind the p27kip1 promoter and repress its activity in the α-T3 pituitary cell line (Li et al., 2002). We observe a downregulation of SIX6, Cyclin D1 and an up-regulation in p27kip1 in *Sox2* mutants. These data all suggest that SOX2 promotes progenitor proliferation at least in part through the upregulation of SIX6. In turn SIX6 may induce proliferation and cell cycle progression through the inhibition of p27kip1. Therefore the genetic interaction we observe between SOX2 and p27kip1 could be indirect, and mediated by SIX6 (Figure 6.1).

By the end of gestation SOX2 and SIX6 co-localise to the MC layer of the AP, in the region in which adult SC reside (data not shown). This suggests that SIX6 may play a role post-natally in adult pituitary SC. p27kip1 is ubiquitous in the adult pituitary (Bilodeau et al., 2009). It has been shown to repress SOX2 *in vitro*, and *p27kip1* null pituitaries show in particular an enlarged $SOX2^{+ve}$ progenitor layer (Li et al., 2012). Therefore a Sox2-Six6-p27kip1 interaction may also regulate SC proliferation/quiescence in the adult and the balance between the different partners is likely to be important. Detailed analysis of expression patterns of the three proteins is required while their function can be approached *in vitro*, using pituispheres.

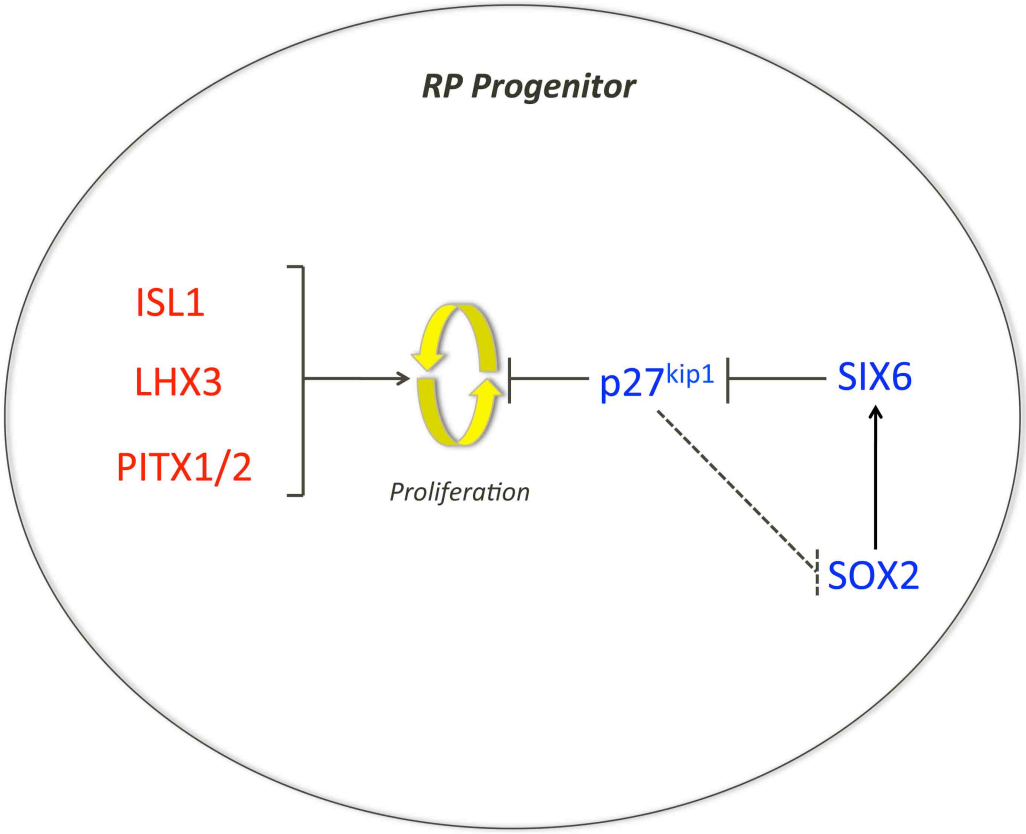

Figure 6.1: Regulation of RP progenitor proliferation.

RP progenitor proliferation is regulated by independent pathways. SOX2 and SIX6 may interact to promote proliferation, at least in part thought inhibition of p27kip1. Other factors promoting progenitor proliferation and maintenance comprise ISL1, LHX3 and PITX1/2.

6.2.3 Proliferation of RP progenitors is regulated independently by different TF cascades

A rudimentary pituitary develops despite the significant reduction of proliferation observed following deletion of *Sox2* throughout RP. It is possible that SOX2 was not deleted early enough to completely inhibit pituitary formation. This is however unlikely as *FoxG1^{Cre}* is active in the anterior neural ridge at 8.5dpc (Wang et al., 2010), in the region where the hypophyseal placode forms. A second and more likely explanation is the continued expression of at least LHX3, ISL1 and PITX1 in *Sox2* mutants (this thesis and Jayakody et al., (2012), three TFs required respectively for progenitor maintenance (LHX3) and proliferation (Zhao et al., 2006, Ellsworth et al., 2008, Takuma et al., 1998). Thus these TFs may account for the reduced level of proliferation and consequently rudimentary RP formation. It is not known if SOX2 and SIX6 expression patterns are maintained in embryos with null mutations for LHX3, ISL1 or PIXT1. However, our data suggest that several TF cascades, acting independently, may regulate proliferation of RP progenitors (Figure 6.1).

6.3 SOX2 and endocrine cell differentiation

Following the initial phase of proliferation and progenitor expansion, RP cells exit the cell cycle, mainly between 11.5dpc – 13.5dpc (Davis et al., 2011) and begin terminal differentiation. Downregulation of SOX2 and cell cycle exit are also associated with the upregulation of TFs that promote lineage commitment and differentiation of the different endocrine cell populations. These include PAX7, TPIT, SF1 and PIT1, (Figure 6.2) (for review see Davis et al., 2013). Cell cycle exit and endocrine cell differentiation are thought to be regulated separately, as preventing cell cycle exit does not inhibit differentiation (Bilodeau et al., 2009).

6.3.1 Anterior lobe

The early hypoplasia following *Sox2* deletion using *FoxG1^{Cre}* results in the loss of nearly all endocrine cell types. Interestingly, the first cell type to exit the cell cycle, the future rostral-tip thyrotrophs (Jean et al., 1994), is also the least affected. All other cell types, that exit concomitantly mainly between 11.5dpc – 13.5dpc (Davies et al., 2011), fail to differentiate. Therefore the cells that exit the cell cycle earlier are less affected. This was also the conclusion reached by Jayakody et al (2012) from their data.

One exception to this rule in Jayakody et al (2012) was the unexpected maintenance of PrL expression, despite the complete loss of PIT1. Similarly, PIT1 is completely downregulated when *Sox2* was deleted using *FoxG1^{Cre}*, but in contrast there was no PrL expression. PROP1 is also downregulated following *Sox2* deletion, however its expression is not completely lost (Jayakody et al., 2012). PROP1 promotes the emergence of gonadotrophs and the PIT1 lineage, but its function in the early RP is unclear as it is mainly co-expressed with SOX2 from 10.5dpc, becoming progressively restricted to the dorsal region of RP (Dasen et al., 1998, Yoshida et al., 2011). Its downregulation may be a contributing factor in the loss of PIT1 and endocrine cell differentiation in the AP. These studies, combined with the results presented in this thesis suggest SOX2 driven proliferation is required to produce a large pool of progenitors that can then progress toward endocrine cell commitment and differentiation.

6.3.2 Intermediate lobe

The phenotype observed in the IL initially appears similar to that observed in the AP. *Sox2* deletion results in severe hypoplasia and a loss of cell identity as the majority of remaining cells persist as POMC^{-ve} non-cycling progenitors. More detailed analysis reveals a complete downregulation of lineage specifier PAX7, resulting in a change of cell fate from GR^{-ve} melanotrophs to GR^{+ve} corticotrophs in the remaining POMC^{+ve} population.

This suggests that SOX2 plays an additional role in the IL and that it is necessary for melanotroph lineage specification (Figure 6.2). This correlates with a different expression pattern of SOX2 in the IL compared to the AP (Figure 6.3). Weak nuclear SOX2 is observed throughout the IL up until adulthood in the region of PAX7 and POMC expression, while cells lining the cleft show strong SOX2 expression (Figure 6.2, 6.3). Nuclear SOX2 is never seen in the AP, although very infrequent hormone^{+ve} cells can have SOX2 protein localised in the cytoplasm, (Fauquier et al., 2008; Gremeaux et al., 2012). This indicates that SOX2 marks two populations of cells in the pituitary; terminally differentiated melanotrophs (SOX2^{LO}) and SC/progenitors (SOX2^{HI}). Similarly adult NSC can be classified into SOX2 Hi and Lo cells, representing spatial and functional differences (Hutton & Pevny., 2011). Loss of PAX7 in *Sox2* mutant IL cells and their subsequent identity switch leads us to hypothesise that SOX2 may directly regulate PAX7 expression. Analysis of the *Pax7* locus did not reveal any SOX2 TFBS and the factors involved in *Pax7* transcriptional regulation in IL are still unknown (J. Drouin personal communication), but this needs to be explored further.

SOX2 and PAX6 are known to physically interact to activate expression of *δ1-crystallin* in the lens placode (Kamachi et al., 2001). PAX7 shares extremely high homology with PAX6 and indeed both contain a PD, which interacts with the HMG domain of SOX2 to form a complex on the *δ1-crystallin* promoter (Kamachi et al., 2001). The SOX2-PAX6 complex can also actively promote the expression of *Pax6* itself. In addition, SOX2 can upregulate *Pax6* expression in head ectoderm (Aota et al., 2003). Based on these data, it would be of interest to investigate potential interactions between SOX2 and PAX7 and whether a SOX2-PAX7 complex participates in *Pax7* regulation. Such an interaction may explain the loss of PAX7 expression in mutants. Deletion of *Sox2* in POMC^{+ve} cells will also allow us to further investigate its role in melanotroph specification and its involvement in PAX7 expression.

In summary we demonstrate a requirement for SOX2 in the dorsal region of RP for proper formation of IL. Our data suggest that in addition to its proliferative role, SOX2 is required here for lineage specification as a switch of endocrine cell identity is observed in its absence.

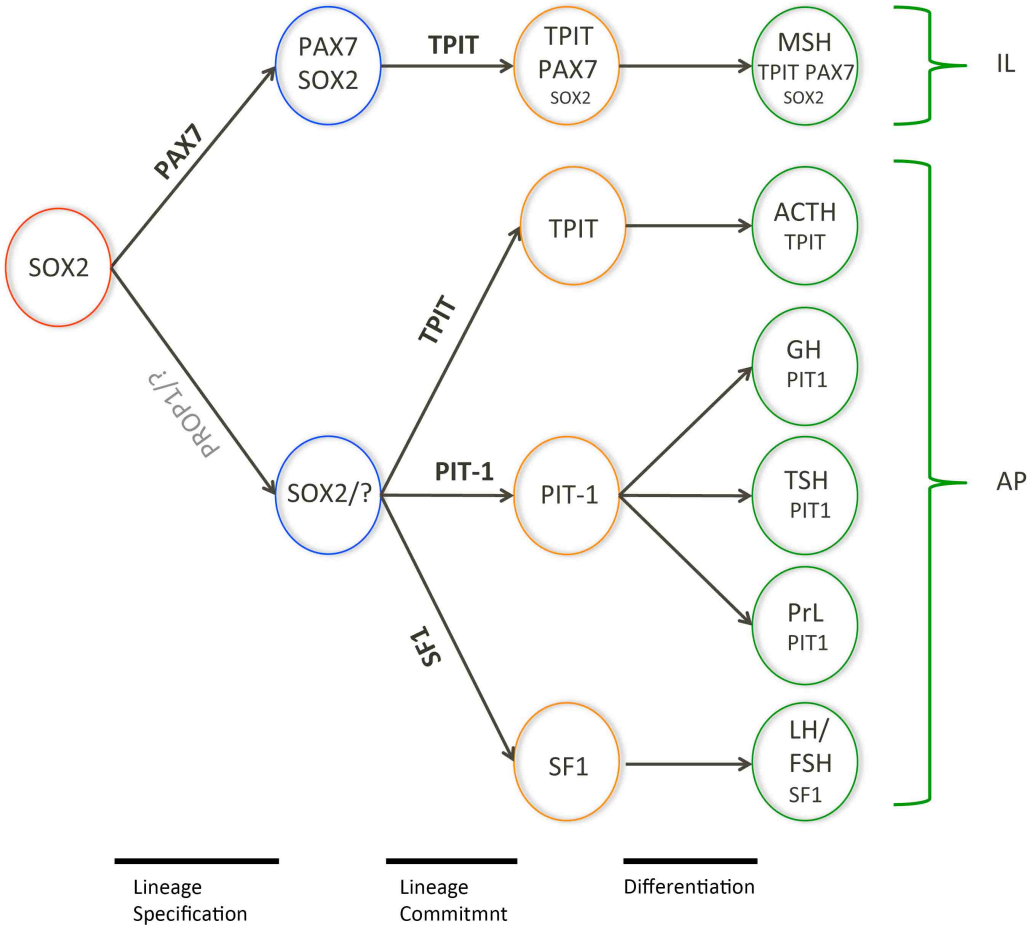

Figure 6.2: Emergence of endocrine cell lineages during pituitary development.

All terminally differentiated endocrine cells are derived from SOX2^{+ve} progenitors. SOX2 and PAX7 co-localise in the dorsal RP to specify the IL melanotroph lineage. SOX2 expression remains in in TPIT melanotrophs however its level of expression is reduced. An unknown factor may perform a similar role to PAX7 in the AP. Following IL and AP lineage specification each individual endocrine cell type undergoes lineage commitment following the expression of lineage markers TPIT, PIT1 and SF1. Finally all endocrine cells differentiate.

6.4 SOX2 regulation of early progenitor proliferation and later IL/melanotroph specification is independent

As suggested earlier, reduction in AP endocrine cell numbers may solely be a consequence of a reduced progenitor pool (see above; Jayakody et al., 2012). This suggests that restoration of proliferation in mutants should also promote differentiation of more endocrine cells. However, at least in the dorsal RP, restoration of proliferation in *Sox2;p27kip1* mutants did not rescue the differentiation defect.

Independent regulation of RP progenitor proliferation and differentiation has been suggested previously (Bilodeau et al., 2009). In this study, differentiation was un-perturbed following the homozygous deletion of 27kip1, p57kp2 or both together, despite the increase in proliferation. We observe a converse situation in our model: restoration of proliferation does not rescue defective differentiation in the absence of SOX2. This observation also suggests that proliferation and differentiation are regulated independently, and moreover that SOX2 may be involved in both processes in the IL, where its expression is normally maintained.

In the AL, the situation is different as the SOX2 is down-regulated when cells differentiate. However, SOX2 may also play an additional role and allow progenitors to become "competent" for commitment and differentiation, possibly through the upregulation of PROP1 (see above). Restoration of proliferation using CDKI null mutants (Bilodeau et al., 2009) on a $FoxG1^{Cre}$; $Sox2^{fl/fl}$ may answer this question.

Classically, SOX2 exerts its function only in partnership with another TF (for review see Kondoh & Kamachi., 2010). Interaction with a partner such as PAX6 in the lens placode is associated with acquisition of lens identity, through the activation of new molecular cascades and in this instance promotion of lens differentiation (Kamachi et al., 2001). Transition in cell fate can be caused by changing one of the two TF in the complex, for

example neural stem cells are characterised by the presence of SOX2/BRN2 complexes, while transition to neural progenitors can be associated with the formation of a SOX11/BRN2 complex (Tanaka et al., 2004). These studies are consistent with a change in fate (and the regulation of relevant processes) being induced through pairing a common TF with different co-factors. Here SOX2 may have different partners to regulate proliferation and differentiation. PAX7 is a likely candidate for forming a complex in the dorsal region of RP to promote IL fate. SOX2 is also capable of forming complexes with members of the SIX family of TF, notably this has been reported for SIX1 (Ahmed et al., 2012). Moreover SOX2 and SIX3 can both bind the *Shh* SBE2 forebrain enhancer, indicating they could possibly form a complex and co-operate to promote *Shh* expression (Jeong et al., 2008; Li Zhao et al., 2012). Thus SOX2 may also promote proliferation co-operatively with SIX6 in addition to activating its expression. The switch between SIX6 and PAX7 in dorsal RP may be crucial for a change in cell fate from a proliferative progenitor to a future melanotroph.

6.5 Model of the role of SOX2 during pituitary development

Based on the data presented in this thesis and previously published studies, we can now propose a combined model of the roles of SOX2 in pituitary development, (Figure 6.3).

SOX2 and SIX6 are expressed throughout RP during its early proliferative phase, prior to cell cycle exit at 11.5dpc (Davis et al., 2011). SOX2 promotes proliferation (this thesis and Jayakody et al., 2012), at least in part through the upregulation of SIX6 and repression of p27kip1. This early SOX2;SIX6 dependent proliferation allows the production of sufficient progenitors to permit both early and late cell cycle exiting cells to be generated, and undergo terminal differentiation. This initial proliferative role of SOX2 in RP is essential to prevent hypoplasia and cellular arrest in a non-cycling progenitor-like state.

Following the dorsal restriction of SOX2 (Fauquier et al., 2008) to the future IL, PAX7 starts to be expressed in these then slow-proliferating cells (Bilodeau et al., 2008; Langlais et al., 2012). In this later phase starting at 14.5dpc, upon the onset of PAX7 expression, SOX2 is required for *Pax7* expression, either directly or indirectly, and this is necessary for IL melanotroph lineage specification (Figure 6.3). An interaction between both proteins may occur in $POMC^{+ve}$ cells. Importantly while SOX2 is involved in both processes, their regulation occurs independently.

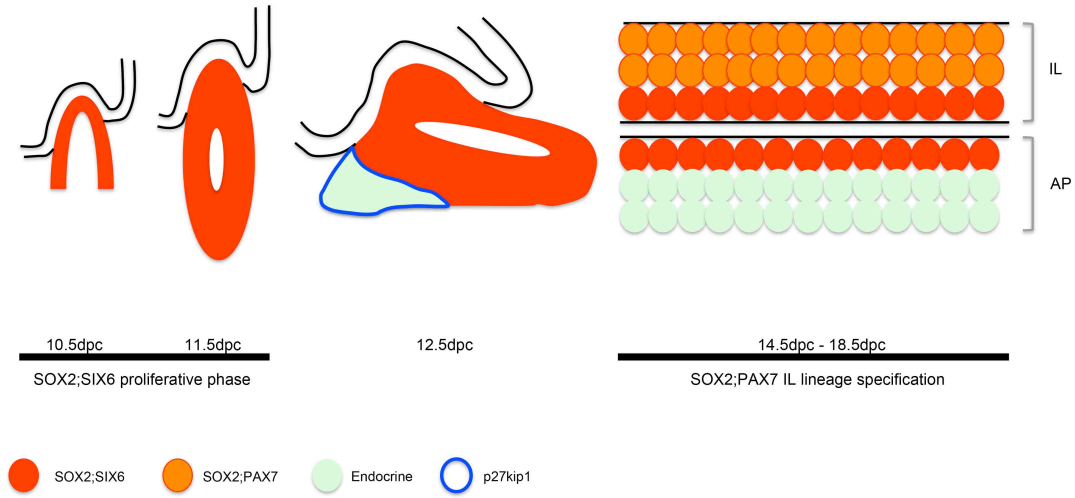

Figure 6.3: Combined model of the role of SOX2 in pituitary development.

SOX2 and SIX6 are ubiquitously expressed in RP during the proliferative phase between 10.5dpc - 11.5dpc. By 12.5dpc endocrine cells have exited the cell cycle and begun to differentiate ventrally. p27kip1 is inhibited in the dorsal region of RP at 12.5dpc to 14.5dpc. At 14.5dpc - 18.5pdc PAX7 is expressed in the dorsal of RP with SOX2, specifying this tissue as IL. $SOX2^{+ve}$ cells lining the lumen of the IL are $PAX7^{-ve}$. IL = intermediate lobe, AP = anterior pituitary.

6.6 Summary

In summary, our results show that SOX2 is necessary for the proliferation of early RP progenitors. It is likely that SOX2 regulates proliferation through the upregulation of SIX6, whose expression is down-regulated upon *Sox2* deletion. Moreover, we demonstrate a genetic interaction between SOX2 and p27kip1, in the dorsal region of RP suggesting that SOX2 may repress p27kip1 expression and this may, in part, be necessary for proliferation. Furthermore, our results show that SOX2 is necessary for PAX7 expression in the dorsal RP and IL where SOX2 is ubiquitously expressed. Loss of PAX7 expression due to *Sox2* deletion, results in a switch in cell fate in the IL, from melanotrophs to corticotrophs, demonstrating a role for SOX2 in IL melanotroph lineage specification. In addition we provide evidence that these two processes regulated by SOX2 in the developing pituitary are independent, as rescuing the proliferation defect in *Sox2* deleted mice does not rescue differentiation. Therefore SOX2 has a dual role in pituitary development and this is likely to depend on alternative binding partners and therefore target genes, that will be identified in future studies.

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